DOES NOT CIRCULATE

OCTOBER 1952, VOLUME 52, No. 2

UNIVERSITY
NOV 4 1952
MEDICAL
LIBRARY

THE BIOCHEMICAL JOURNAL

Edited for

THE BIOCHEMICAL SOCIETY

Editorial Board

R. K. CALLOW
R. E. DAVIES
D. HERBERT
R. A. KEKWICK
W. KLYNE
C. LONG
M. G. MACFARLANE
N. H. MARTIN
R. A. MORTON
A. NEUBERGER (*Chairman*)
A. G. OGSTON
A. PIRIE
R. L. M. SYNGE
T. S. WORK

CAMBRIDGE UNIVERSITY PRESS
LONDON: BENTLEY HOUSE, N.W. 1
NEW YORK: 32 EAST 57TH STREET, 22

Price 20s. net

The *Biochemical Journal* is conducted by the **Biochemical Society** and is published by the Cambridge University Press. The *Journal* is issued in multiple volumes per year. Each volume consists of approximately 640 pages, published in four parts at intervals of one month.

The Biochemical Society was instituted solely for the advancement of the Science of Biochemistry, and holds meetings at different centres for the communication of original papers, and for the discussion of current problems. Persons interested in Biochemistry are eligible for election. Members whose subscriptions are not in arrears are entitled to receive the *Biochemical Journal* without further payment. Further information may be obtained from the Hon. Secs., Professor Leslie Young, St Thomas's Hospital Medical School, London, S.E. 1, and Professor R. H. S. Thompson, Department of Chemical Pathology, Medical School, Guy's Hospital, London, S.E. 1, or from the Hon. Treas., F. A. Robinson, Esq., c/o Allen and Hanburys, Ltd., Bethnal Green, London, E.C. 2.

Subscriptions to the *Biochemical Journal*. For non-members of the Biochemical Society the subscription to the *Biochemical Journal* is £3. 10*s*. 0*d*. per volume, payable in advance to the Cambridge University Press, Bentley House, 200 Euston Road, London, N.W. 1, to F. A. Robinson, Esq., c/o Allen and Hanburys, Ltd., Bethnal Green, London, E.C. 2, or to any bookseller. In the United States of America the *Journal* may be purchased from the Cambridge University Press American Branch, 32 East 57th Street, New York, 22 (subscription price $12.25 per volume).

Communications respecting the sale of single issues or back numbers of the *Journal*, Volumes 20–49, should be addressed to the Cambridge University Press, Bentley House, 200 Euston Road, London, N.W. 1. Volumes 1–15 have been reproduced and are available from Messrs William Dawson & Sons, Ltd., 102 Wigmore Street, London, W.1, at the prices given below.

Claims for the replacement of *Journals* lost in transmission will not be entertained if they are received later than three months after the date of the posting of the *Journal*.

Prices of back numbers of the *Journal*.

Volume 1–15 complete.	£82. 10*s*. bound, £75 unbound
Separate volumes.	£6 each bound, £5. 10*s*. each unbound

Reduced terms for Volumes 1–15 are available to members of the Biochemical Society, who should apply to the Honorary Secretaries.

	In paper cover (except Vol. 38)
Volumes 20–28.	£8 per volume of 6 parts at £1. 12*s*. each if sold separately
Volumes 29–35.	£8 per volume of 12 parts at 16*s*. each if sold separately
Volume 36.	£5 per volume of 12 parts at 10*s*. each if sold separately
Volumes 37 and 40.	£5 per volume of 6 parts at £1 each if sold separately
Volumes 41–43	£5 per volume of 4 parts at £1. 10*s*. each if sold separately
Volumes 38, 39, 44–49.	£5 per volume of 5 parts at £1. 4*s*. each if sold separately
Index to Volumes 11–20.	10*s*. net
Index to Volumes 21–30.	15*s*. net
Index to Volumes 31–40.	£1. 15*s*. net
History of the Biochemical Society	2*s*. 6*d*. net

Binding. Quotations can be given by the publishers for bound copies of back numbers; also for buckram binding cases, and for binding subscribers' sets. (Suspended.)

CONTENTS OF VOLUME 52, No. 2, 1952

(*All rights reserved*)

PAGE

Studies of sebum. 2. Some constituents of the unsaponifiable matter of human sebum. By R. M. B. MACKENNA, V. R. WHEATLEY and A. WORMALL. (With two figures) 161

Spectrophotometric studies on human sebum. By G. N. FESTENSTEIN and R. A. MORTON. (With twelve figures) 168

The reactivity of radiomimetic compounds. 1. Cross-linking of proteins. By P. ALEXANDER, M. FOX, K. A. STACEY and L. F. SMITH. (With two figures) 177

The effect of fluoride on succinic oxidase system. By E. C. SLATER and W. D. BONNER, jun. (With eight figures) 185

The action of thrombin on fibrinogen. By L. LORAND and W. R. MIDDLEBROOK 196

Fibrino-peptide. By L. LORAND. (With one figure) 200

The branched-chain fatty acids of mutton fat. 1. The isolation of (+)-14-methylhexadecanoic acid. By R. P. HANSEN, F. B. SHORLAND and N. J. COOKE 203

Seasonal variations in fatty acid composition of New Zealand butterfat. By R. P. HANSEN and F. B. SHORLAND 207

The biosynthesis of protein. 1. The uptake of glycine, serine, valine and lysine by the mammary gland of the rabbit. By P. N. CAMPBELL and T. S. WORK. (With five figures) 217

Addendum—The quantitative estimation of glycine in small samples of proteins. By S. KROL. (With two figures) 227

Anaerobic and aerobic glycolysis in lactating mammary gland and in nervous tissue. By C. TERNER. (With one figure) 229

Gas-liquid partition chromatography: the separation and micro-estimation of ammonia and the methylamines. By A. T. JAMES, A. J. P. MARTIN and G. HOWARD SMITH. (With six figures) 238

Gas-liquid partition chromatography: the separation of volatile aliphatic amines and of the homologues of pyridine. By A. T. JAMES. (With four figures) 242

Studies in immunochemistry. 11. The isolation and properties of the human blood-group H substance. By E. F. ANNISON and W. T. J. MORGAN. (With six figures) 247

Addendum—Physicochemical examination of blood-group H substance. By R. A. KEKWICK. (With one figure) 259

Citric acid and bone metabolism. By T. F. DIXON and H. R. PERKINS 260

The determination of glucosamine by alkaline decomposition. By M. V. TRACEY 265

The metabolism of methyl carbamate. By E. BOYLAND and D. PAPADOPOULOS 267

Sorbitol antiketogenesis and the dehydrogenation and oxidation of fatty acids. By R. L. BLAKLEY . . 269

Sedimentation and diffusion of human albumins. 2. Nephrotic human albumins at a low concentration. By P. A. CHARLWOOD 279

Studies in the biochemistry of micro-organisms. 89. Metabolic products of *Penicillium multicolor* G.-M. and P. with special reference to sclerotiorin. By J. H. BIRKINSHAW 283

Phosphates of brain during *in vitro* metabolism: effects of oxygen, glucose, glutamate, glutamine, and calcium and potassium salts. By H. MCILWAIN 289

Creatine phosphokinase in mammalian brain. By A. NARAYANASWAMI 295

PAGE

Effects of insulin and of glycerol *in vitro* on the incorporation of [*carboxy*-^{14}C]acetate into the fatty acids of lactating mammary gland slices with special reference to species differences. By J. H. Balmain, S. J. Folley and R. F. Glascock 301

D-Amino-acid oxidase in the molluscan liver. By H. Blaschko and J. Hawkins 306

The effect of age on the bulk protein composition of *Chlorella vulgaris*. By L. Fowden 310

The biosynthesis of L-cystine and L-methionine labelled with radioactive sulphur (^{35}S). By R. B. Williams and R. M. C. Dawson 314

The direct isolation of arginine and lysine picrates from gelatin hydrolysates. By W. Robson and A. S. M. Selim 318

Effect of choline deficiency on the fatty acids of liver and body lipids in the rat. By C. Siva Raman . 320

The effect of light on xanthine oxidase activity. By S. M. Siegel and R. L. Weintraub. (With four figures) 324

A method for the preparation of gastrin. By J. E. Jorpes, O. Jalling and V. Mutt 327

The preparation of secretin. By J. E. Jorpes and V. Mutt 328

The free amino-acids of invertebrate nerve. By P. R. Lewis 330

A method for the quantitative determination in urinary extracts of C_{21} 17:20-dihydroxy-20-methylsteroids. By R. I. Cox. (With one figure) 339

The metabolism of subcutaneously injected [^{14}N] urea in the cat. By H. L. Kornberg and R. E. Davies. (With one figure) 345

The determination of basic groups in collagen and gelatin. By S. C. Ellis and K. G. A. Pankhurst. (With three figures) 350

INDEX OF AUTHORS

	PAGE
Alexander, P.	177
Annison, E. F.	247
Balmain, J. H.	301
Birkinshaw, J. H.	283
Blakley, R. L.	269
Blaschko, H.	306
Bonner, W. D., jun.	185
Boyland, E.	267
Campbell, P. N.	217
Charlwood, P. A.	279
Cooke, N. J.	203
Cox, R. I.	339
Davies, R. E.	345
Dawson, R. M. C.	314
Dixon, T. F.	260
Ellis, S. C.	350
Festenstein, G. N.	168
Folley, S. J.	301
Fowden, L.	310
Fox, M.	177
Glascock, R. F.	301
Hansen, R. P.	203, 207
Hawkins, J.	306
Jalling, O.	327
James, A. T.	238, 242
Jorpes, J. E.	327, 328
Kekwick, R. A.	259
Kornberg, H. L.	345
Krol, S.	227
Lewis, P. R.	330
Lorand, L.	196, 200
McIlwain, H.	289
Mackenna, R. M. B.	161
Martin, A. J. P.	238
Middlebrook, W. R.	196
Morgan, W. T. J.	247
Morton, R. A.	168
Mutt, V.	327, 328
Narayanaswami, A.	295
Pankhurst, K. G. A.	350
Papadopoulos, D.	267
Perkins, H. R.	260
Raman, C. Siva	320
Robson, W.	318
Selim, A. S. M.	318
Shorland, F. B.	203, 207
Siegel, S. M.	324
Slater, E. C.	185
Smith, G. Howard	238
Smith, L. F.	177
Stacey, K. A.	177
Terner, C.	229
Tracey, M. V.	265
Weintraub, R. L.	324
Wheatley, V. R.	161
Williams, R. B.	314
Work, T. S.	217
Wormall, A.	161

Studies of Sebum

2. SOME CONSTITUENTS OF THE UNSAPONIFIABLE MATTER OF HUMAN SEBUM

By R. M. B. MACKENNA, V. R. WHEATLEY and A. WORMALL
Departments of Biochemistry and Dermatology, Medical College of St Bartholomew's Hospital, London, E.C. 1

(*Received* 6 *November* 1951)

Our earlier studies (MacKenna, Wheatley & Wormall, 1950) have shown that human sebum (surface skin fat) contains on an average about 29 % free fatty acids, 36 % combined fatty acids (present in glycerides, waxes and other esters) and 32 % unsaponifiable matter. The unsaponifiable matter contains hydrocarbons, cholesterol and some unidentified compounds, and squalene was identified as one of the major constituents, accounting for about 16 % of the unsaponifiable matter and about 35 % of the hydrocarbon fraction. Some preliminary chemical tests were made for the fat-soluble vitamins, and the presence of a small amount of vitamin E in human sebum was reported.

A more complete study of the unsaponifiable matter has now been made, and this material has been more thoroughly examined for vitamins, especially for vitamin E. A preliminary account of part of this work has already been given (MacKenna, Wheatley & Wormall, 1951).

EXPERIMENTAL

Collection of sebum. Sebum was collected by the method of Ricketts, Squire & Topley (1948, 1951), modified as already described (MacKenna *et al.* 1950). Single specimens were obtained by immersing the forearm of a normal male (age within the range 18–30) in 2 l. of twice-redistilled acetone contained in a tall glass cylinder. At the end of 3 min. the arm was removed from the cylinder, and the other arm extracted for a similar period. The acetone was then distilled off, and the residue of lipid, cellular debris and dirt was extracted with $CHCl_3$; the $CHCl_3$ solution was then filtered and evaporated to dryness and the residue weighed. About 0·1 g. sebum was obtained from each subject.

Bulked collections were obtained from a number of male medical students. In the earlier experiments described here the method used for the collection of single specimens was adopted, the same 2 l. of acetone being used for several extractions up to a maximum of ten. The acetone was then distilled off and the distillate used for a further batch of ten subjects; all the residues from the acetone extracts were pooled and put through the $CHCl_3$ stage together. In later experiments a large glass tank ($24 \times 12 \times 12$ in.; $60 \times 30 \times 30$ cm.) containing 15 l. acetone was used, and with this apparatus two subjects were each able to immerse both their forearms simultaneously.

With some batches of sebum precautions were taken to reduce, as far as possible, the oxidation of readily oxidizable constituents. In these cases the acetone was distilled off under reduced pressure (28–32 cm. Hg) in a stream of N_2 and the residue was extracted with ether instead of $CHCl_3$; the ether was then removed from this extract under reduced pressure at room temperature.

Saponification. This was carried out as recommended by the Society of Public Analysts (Cocks, 1933) with appropriate alterations in the volumes of reagents according to the amount of lipid saponified. With one batch of sebum (*T*) saponification was carried out in the presence of pyrogallol, using the method of Tošić & Moore (1945).

Chromatography. Alumina (British Drug Houses Ltd. 'For chromatography') which had been slightly weakened by storage was used in all experiments except one in which full-strength alumina (Savory & Moore) was used. The solvents used were light petroleum (b.p. 40–60°), 5 % (v/v) and 10 % (v/v) $CHCl_3$ in light petroleum (referred to below as 5 % $CHCl_3$ and 10 % $CHCl_3$, respectively), $CHCl_3$, ether and methanol.

An approximately 1 % solution of the unsaponifiable matter in warm light petroleum was prepared (when this solution was allowed to stand, a fraction tended to separate out and in some experiments this insoluble fraction was examined separately; see below, and also Table 1). This solution was filtered through a column of alumina (for a 1 g. sample a column approximately $1{\cdot}4 \times 10$ cm. was prepared from 20 g. alumina) and the column was then eluted with light petroleum, 5 % $CHCl_3$, 10 % $CHCl_3$, $CHCl_3$ (or ether) and methanol in succession. During the development of the chromatogram a series of bands was obtained which fluoresced in ultraviolet light. In earlier experiments the elution of these bands was followed and various fractions were collected and examined. In later work the eluates were collected in 100 or 150 ml. portions and the weight of dissolved matter in each portion was determined. By plotting these weights graphically the development of the chromatogram could be assessed.

Chemical and physical constants. Iodine numbers were determined in samples of about 3 mg. by the method of Yasuda (1931), and acetyl numbers by the method of West, Hoagland & Curtis (1934) modified so that samples of 30–100 mg. could be analysed. Molecular weights were determined by Rast's camphor method; all other microanalyses were made by Weiler and Strauss of Oxford. All melting points recorded are uncorrected.

Chemical tests for vitamins

Vitamin E. The total $FeCl_3$-reducing substances were determined by the method of Emmerie & Engel (1938, 1939). Attempts were then made to remove non-tocopherols by saponification (Emmerie, 1940), by treatment with 85 % (v/v) H_2SO_4 (Parker & McFarlane, 1940) and by chromatography (Tošić & Moore, 1945). Finally, the effect of

acetylation (Emmerie & Engel, 1943) was tried both on the total unsaponifiable matter and on the fractions obtained in the chromatographic separation (method of Tošić & Moore, 1945). In all these experiments control tests were made with sebum to which had been added a small known amount of α-tocopherol.

Other vitamins. The colorimetric Carr & Price (1926) test was used for vitamin A, but since this invariably gave turbid solutions when applied to sebum, tests were also made with activated dichlorohydrin (Sobel & Snow, 1947) which gave no turbidity. Rosenheim's (1929) test with 90% trichloroacetic acid was used for ergosterol and similar provitamins D; according to Schoenheimer & Evans (1936) 7-dehydrocholesterol and 22-dihydroergosterol give positive reactions in this test.

The colorimetric test of Irreverre & Sullivan (1941) was used to test for vitamin K. It is claimed that this test is specific for 2:3-dialkyl-substituted α-naphthaquinones and that it is not given by the synthetic substitute, 2-methyl-1:4-naphthaquinone. It was not possible for us to carry out control tests with sebum to which had been added the natural vitamin, for our efforts to obtain a pure sample of this vitamin were unsuccessful.

Tests were also made for the presence of two water-soluble vitamins, in case these vitamins were secreted in the sweat and carried over into the sebum. The fluorimetric thiochrome method was used for thiamine, and a photometric 2:6-dichlorophenolindophenol method for ascorbic acid. In these two cases the tests were made on acid extracts of sebum, using 0·1 N-HCl and 10% (v/v) acetic acid, respectively.

Wherever possible control tests were also made with sebum containing added vitamin, in order to assess the sensitivity of the tests and the possibility of interference by other constituents of the sebum.

RESULTS

Properties of fractions obtained by chromatography

Eight batches of sebum (*D*, *E*, *F*, *H*, *K*, *S*, *T* and *U*, obtained, respectively, from groups of 25, 82, 22, 23, 55, 60, 15 and 43 normal medical students) were fractionated by the chromatographic method. Five principal fractions were obtained from each batch, and details of the relative amounts of the fractions obtained from each of the six principal batches are given in Table 1. The fractions from each batch were studied separately and the composite results of these studies are as follows.

Fraction I. This fraction was a white or pale-yellow semi-solid wax composed of hydrocarbons, with an average iodine number of 130–170. Squalene accounted for 30–40% of this fraction. Recrystallization of the crude fraction from ethanol gave a solid hydrocarbon, m.p. 57·6 and 56·2°; mol.wt. (Rast) 428. In order to decide whether this solid hydrocarbon was a normal or a branched-chain paraffin the infrared absorption of a sample was examined (curve *A*; Fig. 1). This showed that this substance is a normal-chain paraffin, and it is suggested that it is probably pentacosane (m.p. 55·5–56·0°; Heilbron, 1936), or a mixture of closely related paraffins.

Table 1. *Chromatographic fractionation of the unsaponifiable matter of six batches of mixed human sebum, using alumina*

	Batch					
Fraction*	*E* (%)	*F* (%)	*H* (%)	*K* (%)	*S* (%)	*T* (%)
I	45·6	37·5	37·3	39·7	30·0	40·3
II	17·6	21·6	23·1	23·7	25·1	18·6
III	13·8	16·3	14·8	13·7	18·8	16·1
IV	4·0	7·1	10·8	6·2	11·8	6·4
V†	2·2 (6·1)‡	6·3	6·4	9·1	6·2 (12·6)‡	13·2

* For details of the separation of these fractions see the text.
† This fraction contained a small amount (about 5% of its weight) of an aluminium compound.
‡ Including the material insoluble in light petroleum.

The crude hydrocarbon fraction gave an immediate green coloration which slowly changed to brown in the Liebermann-Burchard test; this suggested the presence of substances other than paraffin and squalene (e.g. *tetracyclo*squalene; cf. Sobel, 1949). In an attempt to obtain further information about this fraction the infrared spectrum of the material which remained after removal of the solid hydrocarbon was examined. The result (curve *B*; Fig. 1) was, however, not conclusive.

After the hydrocarbon fraction had been eluted from the column a band of yellow pigment followed. At first it was suspected that this compound was a carotene, but further tests showed that it was not of this type. Its nature has not yet been established.

Fraction II. The crude fraction was a pale-yellow wax, m.p. 48–52° and iodine number 40. It gave an unusual yellow coloration with a green fluorescence in the Liebermann-Burchard test. Refractionation on full-strength alumina yielded two fractions; a white, saturated wax, m.p. 49–50°, and a pale-yellow, viscous oil. The white wax on recrystallization from acetone gave a substance, m.p. 62·5°, acetyl number 167, which yielded an

acetyl derivative with m.p. 48·3°. The same substance (m.p. 64 and 65°; acetyl derivative, m.p. 48·8°) was obtained by crystallizing the crude fraction from acetone without preliminary rechromatography. (Found: C, 79·2; H, 13·5. Calc. for $C_{20}H_{42}O$ (eicosanol): C, 80·5; H, 14·1%.) It is thought that this substance is eicosanol (m.p. 65·5–66·5° (Levene & Taylor, 1924), 70° (Ameseder, 1907); acetyl number, 168; acetyl derivative, m.p. 44° (Ameseder, 1907)), but it has not yet been characterized to our satisfaction.

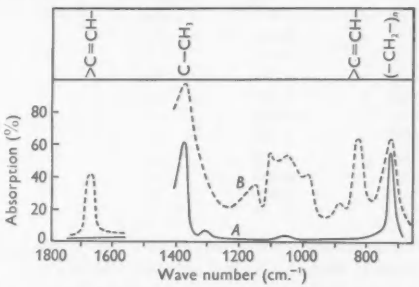


Fig. 1. Infrared absorption spectra of fractions *A* (——, in CS_2 solution) and *B* (- - - -, liquid film 50 μ. thick) from the hydrocarbons (fraction I, Table 1) of the unsaponifiable matter of human sebum. *A* contains the solid saturated hydrocarbons and *B* the residue of fraction I after removal of *A*. (Measurements made by Dr H. P. Koch and Miss J. M. Fabian, using a Grubb-Parsons single-beam instrument.)

The yellow viscous oil obtained on rechromatographing the crude fraction gave the above-mentioned unusual coloration in the Liebermann-Burchard test. This material on analysis gave C, 75·1; H, 11·9; mol.wt. (Rast) 497 and 477 (calc. for $C_{30}H_{50}O_4$: C, 75·9; H, 10·6%; mol.wt. 474) and it appeared to be identical with the primary thermal oxidation product of squalene described by Bolland & Hughes (1949).

Fraction III. This fraction consisted of practically pure cholesterol with traces of pigments. Recrystallization from ethanol gave typical cholesterol crystals, m.p. 148°, and an acetyl derivative, m.p. 114°, was also prepared. Both the crude fraction and the recrystallized material gave a negative Rosenheim (1929) test.

Fraction IV. The crude fraction was a brown gum which gave a positive Liebermann-Burchard reaction, and an immediate brown coloration which slowly changed to green or blue-green in the Rosenheim test. Recrystallization from acetone yielded a small amount of a dirty-white solid, m.p. 76–77° (another sample gave m.p. 95°). Treatment of the crude fraction with a solution of digitonin in methanol yielded a copious precipitate apparently of unchanged digitonin. The material not precipitated by digitonin was a brown gum and gave positive Liebermann-Burchard and Rosenheim tests. The colour obtained in the latter test was qualitatively different from that produced by ergosterol, an observation which was confirmed by the spectrophotometric investigations of Festenstein & Morton (1952). Mr G. N. Festenstein in Prof. Morton's laboratory kindly examined the ultraviolet absorption spectrum of this material and found no evidence of provitamins D_2 and D_3 (see Festenstein & Morton, 1952). If sebum contained such provitamins it is probable that the bulk of them would be in this fraction. This fraction thus contains an unidentified sterol or sterol-like substance which is not precipitated by digitonin and is difficult to crystallize.

Fraction V. This fraction was a dirty-white wax-like substance which gave a brown coloration in the Liebermann-Burchard test. Recrystallization from methanol gave a white amorphous substance, m.p. 79–81° and 78–82°; mol.wt. (Rast) 717.

Fraction insoluble in light petroleum. This fraction on recrystallization from methanol gave a white wax, m.p. 78–79°, which on analysis gave C, 73·1; H, 12·1%; mol.wt. (Rast) 746. The nature of this fraction, which appears to be identical with fraction V, has not been established, but it is believed to contain oxidation products of squalene.

Chromatography of oxidized squalene. Some of the above-mentioned fractions almost certainly contained oxidation products of squalene, and in order to obtain information about the behaviour of these compounds on alumina columns a sample of squalene that had undergone autoxidation was fractionated by the chromatographic method.

Unchanged squalene (28% of the total material) was eluted first (i.e. corresponding to fraction I). Then came a colourless oil (7%) corresponding to fraction II; this gave the yellow-brown coloration with green fluorescence in the Liebermann-Burchard reaction. A yellow viscous oil (35·4%) was eluted with $CHCl_3$ (corresponding to fraction IV of sebum) and this gave a dark-brown coloration in the Liebermann-Burchard test. The remainder of the material was eluted slowly with methanol (corresponding to fraction V of sebum), and consisted of a dirty-white substance which gave a dark brown Liebermann-Burchard reaction. It would appear, therefore, that fractions II, IV and V of sebum might be expected to contain any squalene oxidation products present in the unsaponifiable matter of sebum.

In order to decide whether these oxidation products were present in sebum prior to collection or were produced during the subsequent manipulations, one batch of sebum (*T*) was collected under conditions designed to reduce oxidation to a minimum. The acetone was removed by distillation in N_2 and under reduced pressure, on the day of collection, and the sebum was then saponified in the presence of pyrogallol using the method of Tošić & Moore (1945). Oxidation products of squalene were still apparently present, and fraction II still gave the peculiar yellow-brown Liebermann-Burchard coloration.

From the results of these experiments it is concluded that some oxidation of squalene takes place during the normal removal of acetone and subsequent saponification, but that significant amounts of squalene oxidation products are present in the sebum prior to its removal from the skin.

practically all the $FeCl_3$-reducing substances from sebum, but added α-tocopherol was also destroyed; this modified method is, therefore, useless for tocopherol determinations on sebum. With another batch of sebum (*O*) a comparison was made of the amounts of non-saponifiable, acidic and neutral

Table 2. *Tocopherol determinations on human sebum*

Method	Sebum (mg.)	Tocopherol added (mg.)	Tocopherol found (mg.)	Recovery of added tocopherol (%)	Apparent tocopherol content of sebum (mg./g.)
Emmerie (1940) (saponification)	—	0·1	0·048	48	
	200	—	0·085	—	0·42
	200	0·05	0·105	40	
Tošić & Moore (1945) (saponification in the presence of pyrogallol)	—	0·05	0·038	76	
	200	—	0·084	—	0·42
	200	0·05	0·116	64	
Parker & McFarlane (1940) (removal of 'impurities' with 85% (v/v) H_2SO_4)	—	0·06	0·057	95	
	200	—	0·009	—	0·05
	200	0·06	0·020	18	

Vitamin E and other $FeCl_3$*-reducing substances*

One of the most widely used chemical methods for determining vitamin E is that in which the colour given with ferric chloride and 2:2′-dipyridyl is measured colorimetrically (Emmerie & Engel, 1938, 1939). Determination of the total $FeCl_3$-reducing substances in sebum by this method showed that appreciable amounts (0·8–3·3 mg./g. sebum, calculated as α-tocopherol) were present, while recovery of added α-tocopherol was found to

Table 3. $FeCl_3$*-reducing substances in sebum*

	(Calc. as mg. α-tocopherol/g. of sebum)
Total	1·78
Acidic	1·35
Neutral*	0·59

* A similar value (0·60) was obtained with the unsaponifiable fraction of this sebum. This would suggest that the loss of reducing substances during the saponification was balanced by reducing substances liberated by hydrolysis of esters.

be fairly satisfactory (82%). Further study was made of these $FeCl_3$-reducing substances to decide whether, in fact, tocopherols were present. The effect of saponification, and of treatment with 85% (v/v) sulphuric acid, was examined first. The results (Table 2) showed that significant amounts of $FeCl_3$-reducing substances were present in the unsaponifiable fraction, though the control tests showed that appreciable destruction of added α-tocopherol occurred even when the saponification was carried out in the presence of pyrogallol as advocated by Tošić & Moore (1945). Treatment with 85% (v/v) sulphuric acid (Parker & McFarlane, 1940) removed $FeCl_3$-reducing substances present, and it was found (Table 3) that the largest amount was in the acidic fraction, the neutral or unsaponifiable fractions containing only about a third of the total $FeCl_3$-reducing substances. The acidic fraction can be removed by extracting an ethereal solution of sebum with aqueous 1% potassium hydroxide.

Table 4. *Tocopherol determinations on sebum after chromatography and acetylation*

	Unsaponifiable $FeCl_3$-reducing substances	
	Sebum (mg./g. sebum)	Sebum plus α-tocopherol* (mg./g. sebum)
(*a*) Before passing through column		
Before acetylation	0·44	0·85 (recovery 82%)
After acetylation	0·22	0·29
(*b*) After chromatography		
Unadsorbed:		
Before acetylation	0·19	0·46 (recovery 54%)
After acetylation	0·08	0·08
Adsorbed:		
Before acetylation	0·15	0·15
After acetylation	0·0	0·0

* 0·5 mg. α-tocopherol added/g. of sebum.

The unsaponifiable $FeCl_3$-reducing substances were further investigated. The chromatographic method of Tošić & Moore (1945) was used, and determinations were made of the $FeCl_3$-reducing power of the adsorbed and unadsorbed fractions before and after they had been acetylated (tocopherol esters do not reduce ferric ions). In this chromatographic method α-tocopherol is not adsorbed by the column, whereas most of the β-

tocopherol is adsorbed and many interfering substances are completely adsorbed. The results (Table 4) show that the α-tocopherol content of these sebum samples was probably not greater than 0·2 mg./g. sebum, even if allowance is made for a loss of 50 % of the vitamin in the saponification and chromatographic separation. These results suggest that only a trace of vitamin E is present in sebum, but that other $FeCl_3$-reducing substances are present in the unsaponifiable fraction.

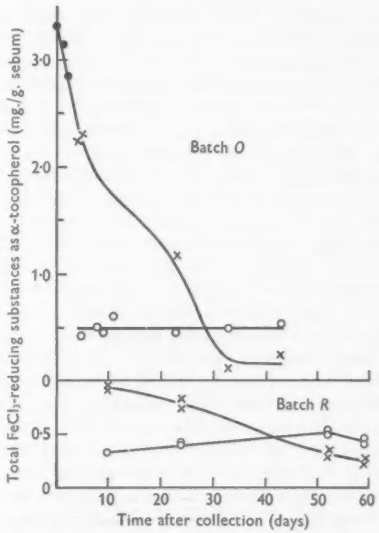


Fig. 2. The effect of autoxidation on the total (×) and non-saponifiable (○) $FeCl_3$-reducing substances of two batches (*O* and *R*) of human sebum. Earlier determinations of the total (●) $FeCl_3$-reducing substances of batch *O* were made on the acetone solution of sebum.

Since appreciable amounts of $FeCl_3$-reducing substances are present in sebum it was thought that they might serve as antioxidants. The effect of autoxidation on the reducing capacity of sebum was therefore studied.

Two batches of sebum were kept at room temperature in closed desiccators containing air. Samples were removed at intervals and determinations of total and unsaponifiable $FeCl_3$-reducing substances were made, the method of Tošić & Moore (1945) being used for the saponification. With one batch of sebum (*O*) the amount of $FeCl_3$-reducing substances in the acetone solution of sebum was determined, without removal of the acetone, immediately after collection and at intervals during the next 3 days.

The results (Fig. 2) show that the acidic $FeCl_3$-reducing material is rapidly oxidized, while the non-saponifiable substance remains constant for many days. The observation that, after prolonged storage of the sebum, the amount of unsaponifiable $FeCl_3$-reducing material is apparently greater than the total, may be accounted for by the liberation of $FeCl_3$-reducing substances during the saponification or to the presence of interfering substances in untreated sebum, or to both factors.

In some preliminary investigations we have fed considerable amounts of mixed tocopherols (the equivalent of 100–300 mg. of α-tocopherol/day, for up to 42 days) to two subjects, in an attempt to increase the excretion of vitamin E in the sebum. It was thought that any evidence of increased secretion of the vitamin might be of interest in view of the fairly frequent administration of relatively large doses of tocopherols to patients suffering from certain skin diseases. The amount of sebum collected from the forearms of these subjects was insufficient for chromatographic treatment of the unsaponifiable matter, and this investigation was limited to a determination of the total $FeCl_3$-reducing substances in the untreated sebum. These values showed considerable fluctuation from day to day, with each of the subjects studied, but the general conclusion drawn from the results was that the administration of large amounts of tocopherols did not lead to any significant increase in the excretion of $FeCl_3$-reducing substances.

Vitamins other than tocopherols

Many samples of fresh sebum collected from individual subjects and also a mixed sample from fourteen subjects were tested for vitamins A, K, B_1 and C and provitamin D, using about 100 mg. of sebum for each test. Tests were also made on specimens containing added vitamin, so that an upper limit could be put to the amount of vitamin that is detectable by these chemical tests under the conditions of our experiments. The results obtained are shown in Table 5, together with the results of

Table 5. *Colorimetric tests for vitamins in sebum*

Vitamin	Calculated limit of detection (per g. sebum)	Amount found (per g. sebum)
A	10 i.u.	None
Pro-D_2 and D_3	0·3 mg.	None
E	0·01 mg.	0·2 mg.
K	0·1 mg.	None
B_1	1·5 μg.	None
C	0·05 mg.	None

the vitamin E determinations. Sebum thus appears to contain little, if any, of the vitamins included in this list. It was not considered, however, that these colorimetric methods were sufficiently reliable or sensitive for these results to be accepted without confirmation, and at our request Prof. R. A. Morton kindly agreed to examine the ultraviolet absorption spectra of several batches of sebum. The results of

these studies, a preliminary report of which has already been made (Festenstein & Morton, 1951), confirm our observations that vitamins A and K, β-carotene and provitamins D_2 and D_3 cannot be detected in human sebum, and that only a trace, if any, of vitamin E is present (cf. also Festenstein & Morton, 1952).

DISCUSSION

In a previous investigation (MacKenna *et al.* 1950) it was found that human sebum contains about 32% of unsaponifiable matter, including 5% of squalene. The results recorded here show that the chief constituents of this unsaponifiable matter are hydrocarbons (30–46%), normal-chain aliphatic alcohols (14–20%) and cholesterol (14–19%). The remainder consists of oxidation products of squalene and several other unidentified substances, including a 'non-cholesterol' sterol-like compound.

Squalene accounts for 30–40% of the hydrocarbons, and the remainder of this fraction is mainly normal paraffins, though evidence suggesting the presence of another unsaturated hydrocarbon besides squalene has been obtained. The presence of hydrocarbons in sebum is of special physiological interest. It is possible that this elimination of hydrocarbons merely represents the excretion of metabolically useless substances, but there is also the possibility that some of them, in particular squalene, are important metabolites. A more complete examination is now being made of hydrocarbon excretion in the sebum by normal individuals and by those suffering from various skin disorders, in order to obtain information about the possible functions of these compounds in skin metabolism. Squalene is present also in ovarian dermoid cyst lipids and other sebum-like materials (Dimter, 1932; Sobel, 1949) and the structural relationship between this hydrocarbon and carotenoids, some of the fat-soluble vitamins (e.g. A and D) and the sterols and steroids should not be overlooked.

Squalene ($C_{30}H_{50}$) contains six double bonds, and it is fairly readily oxidized (Chapman, 1917). Oxidation yields several products, but Bolland & Hughes (1949) found that the chief product formed in the early stages of oxidation by oxygen at 55° is a diperoxide in which four atoms of oxygen are combined with 1 squalene molecule. Little is known, however, of other oxidation products, though it is believed that some have a cyclic structure. Oxidation of squalene is accelerated by ultraviolet irradiation (Farmer & Sutton, 1942), and we were not surprised to find that part of the squalene in human sebum is present in an oxidized form. Sebum forms a thin film on the skin, with an average thickness of about 0·7 μ., calculated from our yields from the forearm, and at temperatures approaching 37°, and with occasional exposure to ultraviolet rays, oxidation of squalene and other unsaturated compounds in sebum will undoubtedly occur. Our results indicate that untreated human sebum contains several oxidation products of squalene, but because of the small amounts present and the relative lack of information about the chemical nature of the oxidation products, we have not yet been able to identify these compounds.

No evidence has been obtained that human sebum contains 'isocholesterol' (lanosterol and agnosterol). These triterpene alcohols apparently occur only in the sebum of certain animals, notably the sheep, goat, llama and dromedary (Lederer & Kiun, 1945). Our observation provides a further example of the considerable differences between the sebum of different species of animals.

Human sebum contains appreciable amounts of substances capable of reducing ferric chloride under the conditions usually adopted in the Emmerie & Engel (1938, 1939) colorimetric method for determining vitamin E, or in various modifications of this method (cf., for example, Emmerie, 1940, 1949; Tošić & Moore, 1945). On the basis of these results, and in view of the fact that this method is widely used for the determination of the vitamin E content of blood, body fats and foods, it was concluded (MacKenna *et al.* 1950) that human sebum contains significant amounts of this vitamin. Our further investigations, in which we have used several methods for excluding non-tocopherol reducing substances, have shown, however, that the amount of vitamin E in sebum is in fact small; it is probably not greater than the amount present in adipose-tissue fat and other body fats. The problem of the chemical assay of vitamin E is 'complicated by the plurality of tocopherols having different activities, by the interference of other inactive compounds, and by the instability of tocopherols during the manipulations' (Eden & Booth, 1950). Thus it seems probable that a reliable value for the vitamin E content of human sebum will only be possible when a large amount of sebum can be collected for the more specific but laborious bioassay. It is of interest, however, that Festenstein & Morton (1951, 1952) find that our samples of human sebum contain little, if any, vitamin E detectable by ultraviolet absorption. We have not yet established the nature of the non-tocopherol substances reducing ferric chloride present in human sebum, but the main constituent of this group is saponifiable and is apparently of acidic (or phenolic) nature. It seems probable that this compound (or compounds) may have a valuable antioxidant effect in sebum, thus retarding the oxidation of important unsaturated fatty acids and any vitamin E present.

An exhaustive examination of human sebum for fat-soluble vitamins other than vitamin E has not been possible, but chemical tests have failed to reveal the presence of any of these vitamins in amounts which would be significant. The absence of vitamin A (and β-carotene) is interesting in view of the function of this vitamin in maintaining normal keratinization of the skin. Our observation in this respect is consistent with the finding of Cornbleet & Popper (1942) that vitamin A cannot be detected in the epidermis of man though significant amounts are present in the adjacent fatty tissues.

Our inability to detect any provitamin D_2 and D_3 in human sebum is of special interest. Provitamin D_3 (7-dehydrocholesterol) has been isolated from pig skin by Windaus & Bock (1937) and has been shown, by ultraviolet spectrum methods, to be present in chicken skin (Koch & Koch, 1941) and in frog ovaries (Morton & Rosen, 1949). Hou (1930) has suggested that in the rat vitamin D is formed in the sebum. Helmer & Jansen (1937) have shown that irradiated human surface skin fat and also the surface skin fat from irradiated human subjects possess antirachitic activity. There is therefore a strong indication that in man vitamin D may be formed in the sebum, but it is difficult to reconcile this theory with the absence of provitamins D_2 and D_3 from human sebum unless the provitamin is another, as yet unidentified, substance. From this point of view the unidentified 'sterol-like' substance in fraction IV is of interest since it gives a positive Rosenheim reaction. Some of the properties of this compound are similar to those recorded for 7α-hydroxycholesterol, but as yet we have been unable to precipitate it with digitonin. A further study of this compound is being made as part of a wider investigation on the mode of formation of vitamin D by the skin.

SUMMARY

1. A study has been made of the unsaponifiable matter of human sebum. Chromatographic analysis yielded hydrocarbons (30–46 % of the unsaponifiable matter), cholesterol (14–19 %), normal-chain aliphatic alcohols (about 20 %) and some unidentified substances including oxidation products of squalene.

2. The hydrocarbon fraction contains 30–40 % squalene, and a normal-chain paraffin (possibly pentacosane).

3. The principal sterol present is cholesterol, but another sterol-like substance is also present.

4. No vitamin A, β-carotene, vitamin K, or provitamins D_2 and D_3 could be detected by chemical tests on sebum or the separated fractions.

5. Sebum contains appreciable amounts of ferric chloride-reducing substances. Part of this material is probably vitamin E, but not more than the equivalent of 0·2 mg. of tocopherol per gram of sebum. The principal reducing substance is an acidic (or phenolic) compound which may function as an antioxidant in sebum.

The authors wish to express their gratitude to Prof. R. A. Morton, F.R.S., and Mr G. N. Festenstein for their collaboration in the ultraviolet spectrum examinations, and to those medical students, technicians, research workers and teaching staff of our College who acted as subjects for these investigations. Thanks are also tendered to the British Rubber Producers' Research Association for generous samples of pure squalene, and to two members of their staff, Dr H. P. Koch and Miss J. M. Fabian, for kindly carrying out the infrared spectrum determinations. We are also indebted to Messrs Glaxo Laboratories Ltd. for a supply of mixed tocopherols. Part of the expenses of the investigation were covered by expenses grants to one of us (A.W.) by the Medical Research Council and the Central Research Fund of London University.

REFERENCES

Ameseder, F. (1907). *Hoppe-Seyl. Z.* **52**, 121.
Bolland, J. L. & Hughes, H. (1949). *J. chem. Soc.* p. 492.
Carr, F. H. & Price, E. A. (1926). *Biochem. J.* **20**, 497.
Chapman, A. C. (1917). *J. chem. Soc.* p. 56.
Cocks, L. C. (1933). *Analyst*, **58**, 203.
Cornbleet, T. & Popper, H. (1942). *Arch. Derm. Syph., Chicago*, **46**, 59.
Dimter, A. (1932). *Hoppe-Seyl. Z.* **208**, 55.
Eden, E. & Booth, V. H. (1950). *Food Manuf.* **25**, 279.
Emmerie, A. (1940). *Rec. Trav. chim. Pays-Bas*, **59**, 246.
Emmerie, A. (1949). *Ann. N.Y. Acad. Sci.* **52**, 309.
Emmerie, A. & Engel, C. (1938). *Rec. Trav. chim. Pays-Bas*, **57**, 1351.
Emmerie, A. & Engel, C. (1939). *Rec. Trav. chim. Pays-Bas*, **58**, 895.
Emmerie, A. & Engel, C. (1943). *Z. Vitaminforsch.* **13**, 259.
Farmer, E. H. & Sutton, D. A. (1942). *J. chem. Soc.* p. 139.
Festenstein, G. N. & Morton, R. A. (1951). *Biochem. J.* **48**, xxxix.
Festenstein, G. N. & Morton, R. A. (1952). *Biochem. J.* **52**, 168.
Heilbron, J. M. (1936). *Dictionary of Organic Compounds*, 1st ed., vol. 2. London: Eyre and Spottiswoode.
Helmer, A. C. & Jansen, C. H. (1937). *Stud. Inst. Divi Thomae*, **1**, 207.
Hou, H. C. (1930). *Chin. J. Physiol.* **4**, 345.
Irreverre, F. & Sullivan, M. X. (1941). *Science*, **94**, 497.
Koch, E. M. & Koch, F. C. (1941). *Poult. Sci.* **20**, 33.
Lederer, E. & Kiun, T. P. (1945). *Bull. Soc. Chim. biol., Paris*, **27**, 419.
Levene, P. A. & Taylor, F. A. (1924). *J. biol. Chem.* **59**, 905.
MacKenna, R. M. B., Wheatley, V. R. & Wormall, A. (1950). *J. invest. Derm.* **15**, 33.
MacKenna, R. M. B., Wheatley, V. R. & Wormall, A. (1951). *Biochem. J.* **48**, xxxviii.
Morton, R. A. & Rosen, D. G. (1949). *Biochem. J.* **45**, 612.

Parker, W. E. & McFarlane, W. D. (1940). *Canad. J. Res.* **18** (B), 405.
Ricketts, C. R., Squire, J. R. & Topley, E. (1948). Personal communication.
Ricketts, C. R., Squire, J. R. & Topley, E. (1951). *Clin. Sci.* **10**, 89.
Rosenheim, O. (1929). *Biochem. J.* **23**, 47.
Schoenheimer, R. & Evans, E. A. (1936). *J. biol. Chem.* **114**, 567.
Sobel, A. E. & Snow, S. D. (1947). *J. biol. Chem.* **171**, 617.
Sobel, H. (1949). *J. invest. Derm.* **13**, 333.
Tošić, J. & Moore, T. (1945). *Biochem. J.* **39**, 498.
West, E. S., Hoagland, C. L. & Curtis, G. H. (1934). *J. biol. Chem.* **104**, 627.
Windaus, D. & Bock, F. (1937). *Hoppe-Seyl. Z.* **245**, 168.
Yasuda, M. (1931). *J. biol. Chem.* **94**, 401.

Spectrophotometric Studies on Human Sebum

By G. N. FESTENSTEIN and R. A. MORTON
Department of Biochemistry, The University of Liverpool

(*Received* 6 *November* 1951)

The absorption spectra of the samples investigated were examined between 230 and 500 mμ., with special reference to the ultraviolet region of the spectrum; in particular it was sought to establish evidence for vitamin E.

α-, β-, γ- and δ-tocopherols show an absorption band near 295 mμ., $E^{1\%}_{1\,cm.}$ about 80. α-Tocopherol acetate shows a maximum at 285 mμ., $E^{1\%}_{1\,cm.}$ about 40. MacKenna, Wheatley & Wormall (1950, 1951, 1952) have found sebum to contain appreciable amounts of $FeCl_3$-reducing substances; part of this material is stated to be vitamin E, the amount not exceeding 0·2 mg. tocopherol per gram of sebum.

The investigations to be described were carried out using sebum samples of about 1 g. A simple calculation shows that under such circumstances detection of tocopherol must be difficult: if the whole amount (0·2 mg.) of tocopherol can be concentrated in a volume of 4 ml. (in a 1 cm. cell) then the extinction value at 295 mμ. will only be 0·4 (by way of comparison as small a quantity as 0·009 mg. vitamin A will give a similar extinction value at its absorption maximum). The chromatographic separation must be such as to concentrate the tocopherol in one fraction. Furthermore, other absorbing materials will have to be eliminated for the maximum at 295 mμ. to be evident.

A preliminary report of this work has already been given (Festenstein & Morton, 1951). Two samples of sebum and sebum unsaponifiable material were investigated. In addition, a further sample of unsaponifiable material, which had been assayed for $FeCl_3$-reducing substances by MacKenna *et al.* was examined; also a 'sterol-like' fraction provided by the same workers.

EXPERIMENTAL

Chromatography. Separations were carried out using Spence's grade 0 alumina (weakened by controlled addition of water in some cases); chromatographic tubes of 9 and 14 mm. internal diameter were used, depending on the amount of material.

The sebum or sebum fraction was usually dissolved in the minimum amount of light petroleum (A.R. quality) and the column developed at first with this solvent alone; any residue was discarded. Mixtures of ether and light petroleum, ether, methanol and ethanol were used as eluants. Arbitrary fractions were collected, but where distinct bands appeared to be eluting the fractionation was extended. All fractions were taken to dryness and weighed before spectroscopic examination; in the later work particularly, evaporation of solvent was carried out in an atmosphere of CO_2 or N_2, to minimize oxidation of any tocopherol.

The ether used was peroxide-free (usually distilled over reduced Fe before use).

Spectroscopy. Absorption spectra were determined quantitatively using a Beckman photoelectric spectrophotometer. Ethanol and *cyclo*hexane were used as solvents. Readings were taken every 5 mμ. in the range 230–310 mμ., and every 10 mμ. in the range 310–500 mμ.

RESULTS

Sebum. The sebum itself showed mainly end absorption, though there was a suggestion of an inflexion between 270 and 300 mμ. (Fig. 1).

Chromatography resulted in a good fractionation (cf. Table 1), but no selective absorption obviously characteristic of vitamins E appeared in any fraction (Figs. 2 and 3). Since, however, fraction 2 (sample 1, Table 1) showed a faint inflexion near 290 mμ., it was rechromatographed (Table 2), and absorption again examined (Fig. 4). No real evidence is found for the presence of tocopherol in any of these fractions. At least a marked inflexion at 290 mμ. should be shown to be indicative of tocopherol.

In view of the low recoveries from the chromatography of the sebum (57 % even after elution with ethanol) it was decided to remove the free fatty acids prior to chromatography, lest these should prevent the elution of any tocopherol.

Table 1. *Chromatography of sebum*

Fraction no.	Eluant	% of original wt.	$E^{1\%}_{1\,cm.}$ 295 mμ.	Character of ultraviolet absorption spectrum
	Sample 1. $E^{1\%}_{1\,cm.}$ 295 mμ. = 3·0. 2 g., on 20 g. alumina column 10 × 1·4 cm.			
1	Light petroleum	27·9	2·4	Inflexion 250–270 mμ.
2	10% ether/p*	8·7	2·3	Inflexions 250–270, 245, 290 mμ. (?)
3	20% ether/p	2·9	4·5	Weak inflexion 260–280 mμ.
4	50% ether/p	7·4	4·5	Mainly end absorption
5	Ether	6·3	4·5	
6	Methanol	3·9	7·3	
		57·1% recovery		
	Sample 2. $E^{1\%}_{1\,cm.}$ 295 mμ. = 2·8. 1·2 g., on 20 g. alumina column 10 × 1·4 cm.			
1	Light petroleum +5% ether/p	30·5	2·6	Inflexion 250–270 mμ.
2	10% ether/p	4·4	2·3	Maximum 258 mμ.
3	50% ether/p	7·5	4·7	Mainly end absorption
4	Ether	13·1	1·8	
5	Ethanol	0·6	41	
6	Ethanol	1·3	5·7	
		57·4% recovery		

* p = light petroleum.

Table 2. *Rechromatography of a sebum fraction*

(0·173 g., on alumina column 4·5 × 1·4 cm.)

Fraction no.	Eluant	% of original wt.	$E^{1\%}_{1\,cm.}$ 295 mμ.	Character of ultraviolet absorption spectrum
1	2% ether/p*	2·9	5·4	Inflexions 235, 275, 305, 250–270 mμ.
2	5% ether/p	6·5	8·0	Weak maxima 233, 255 mμ. Inflexions 245, 280, 290, 205 mμ.
3	10% ether/p	8·1	12	Maximum, 257 mμ. Inflexions 290, 305 mμ.
4	Ether	45·7	1·3	Chiefly end absorption
5	Ethanol	9·8	11	
		73% recovery		

* p = light petroleum.

Table 3. *Chromatography of sebum from which free fatty acids and other materials had been removed*

(0·914 g., on 20 g. alumina column 10 × 1·4 cm.)

Fraction no.	Eluant	% of original wt.	$E^{1\%}_{1\,cm.}$ 295 mμ.	Character of ultraviolet absorption spectrum
1	Light petroleum	39·2	1·5	Weak inflexion 250–270 mμ.
2	10% ether/p*	16·7	1·2	Very weak inflexion 250–270 mμ.
3	50% ether/p	15·4	1·2	Very weak inflexion 260–280 mμ.
4	Ether	13·7	0·93	Mainly end absorption
5	Ethanol	3·0	2·8	
		88% recovery		

* p = light petroleum.

A solution of sebum in light petroleum was shaken with 5% (w/v) Na_2CO_3 solution until no more colour was extracted. The light petroleum fraction was washed free from alkali, dried over anhydrous Na_2SO_4 and taken to dryness.

As a further means of concentration the residue obtained was partitioned between light petroleum and 90% ethanol (after Evans, Emerson & Emerson (1936) who washed a light petroleum solution of wheat-germ oil non-saponifiable material with small volumes of 92% methanol to remove 'xanthophylls and other inert material'). After removal of solvent the light petroleum fraction was chromatographed (cf. Table 3).

The inflexions are not so marked as for the untreated sebum fractions; also the $E^{1\%}_{1\,cm.}$ 295 mμ. values can be seen to be generally lower.

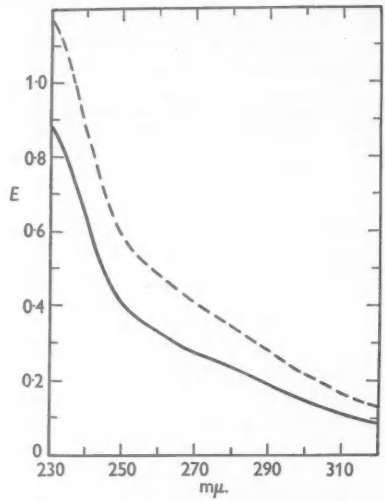


Fig. 1. Absorption spectra of sebum (in *cyclo*hexane). – – –, Sample 1, 0·08 %; ——, sample 2, 0·06 %.

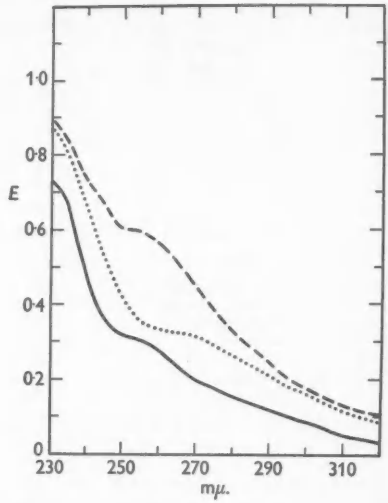


Fig. 2. Chromatographed sebum fractions (sample 1) (in *cyclo*hexane). ——, Fraction 1, 0·042 %; – – –, fraction 2, 0·087 %; · · · · ·, fraction 3, 0·04 %.

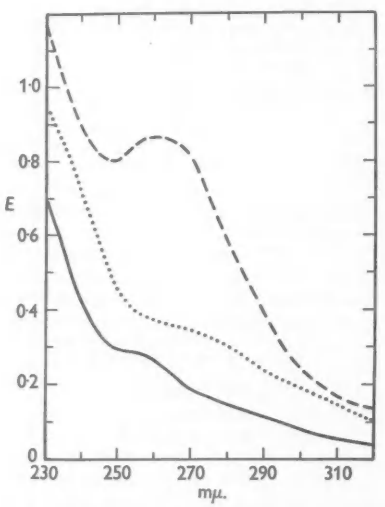


Fig. 3. Chromatographed sebum fractions (sample 2) (in *cyclo*hexane). ——, Fraction 1, 0·037 %; – – –, fraction 2, 0·132 %; · · · · ·, fraction 3, 0·054 %.

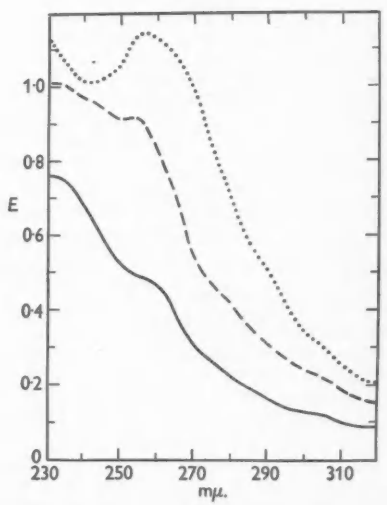


Fig. 4. Rechromatographed sebum fraction 2 (sample 1) (in *cyclo*hexane). ——, Fraction 1, 0·025 %; – – –, fraction 2, 0·033 %; · · · · ·, fraction 3, 0·034 %.

Table 4. *Chromatography of sebum unsaponifiable matter*

Fraction no.	Eluant	% of original wt.	$E^{1\%}_{1\,cm.}$ 295 mμ.	Character of ultraviolet absorption spectrum
Sample 1. 0·5 g., on 20 g. alumina column 10 × 1·4 cm.				
1	Light petroleum	40·2	—	Weak maximum 270 mμ., $E^{1\%}_{1\,cm.}$ 3·1
2	10% ether/p*	1·6	—	Maxima ($E^{1\%}_{1\,cm.}$): 235 mμ. 360; 255 mμ. 335
3	20% ether/p	0·4	—	Faint inflexions 290, 305 mμ. Maximum 258 mμ., $E^{1\%}_{1\,cm.}$ 325 Inflexions 270, 290 mμ.
4	50% ether/p	15·0	4·7	Chiefly end absorption
5	Ether	21·6	5	
6	Methanol	5·4	4	
7	Methanol	0·4	15·8	
		84·6% recovery		
Sample 2. 1 g., on 'weakened' alumina column 12 × 1·4 cm.				
1	Light petroleum	47·3	3·5	Inflexions 250–260, 290 mμ. (?)
2	10% ether/p	7·5	6·2	Inflexion 250–270 mμ.
3	10% ether/p	15·8	3	Mainly end absorption
4	10% ether/p } 20% ether/p }	8·0	3	
5	20% ether/p	1·4	5·3	
6	50% ether/p	0·5	18	
7	Ether	5·0	6	
8	Ethanol	0·5		
		86% recovery		

* p = light petroleum.

Unsaponifiable fraction of sebum. This material (as obtained by MacKenna *et al.*) was examined similarly. The first sample (batch *M*) showed an inflexion near 290 mμ. ($E^{1\%}_{1\,cm.}$ 7·5) consistent with the presence of some vitamin E (Fig. 5). Correction for 'irrelevant absorption' by assuming the latter to be represented by a continuous curve (see uppermost curve, Fig. 5) gives the $E^{1\%}_{1\,cm.}$ 290 mμ. of the selectively absorbing material as about 0·25. It follows that the tocopherol content of the fraction could be of the order 0·3%. The material was chromatographed (Table 4).

Since fractions 2 and 3 showed inflexions at 290 mμ. (Fig. 6), they were rechromatographed, as shown in Table 5.

Fraction 2″ (Fig. 7) exhibits a genuine inflexion at 280–300 mμ. which could be due to a trace of tocopherol, although this is not certainly the absorbing entity.

A second sample of sebum unsaponifiable matter was obtained here by saponification according to the method of Emmerie (1940), who saponified wheat-germ oil under these conditions to obtain 98% recovery of tocopherol. MacKenna *et al.* (1952), using this method on sebum, obtained 40% recovery of added tocopherol; they recommend saponification in the presence of pyrogallol according to the method of Tošić & Moore (1945), when 64% of the tocopherol added to sebum is recovered.

Table 5. *Rechromatography of fractions* 2 *and* 3

Fraction 2 (0·008 g.) on alumina column 4 × 0·9 cm.

Fraction no.	Character of ultraviolet absorption spectrum
1′	Maximum 232 mμ.; feeble inflexion 258 mμ.
2′	Maximum 235 mμ.; inflexions 245–260, 285 mμ.
3′	Maxima 240, 260 mμ. (weak)
4′	Maximum 258 mμ.; feeble inflexions 280, 290 mμ.
5′	Maxima 255, 261 mμ.

Fraction 3 (0·002 g.) (Table 4) plus 4′ from the previous chromatography were rechromatographed on an alumina column 3 × 0·9 cm.

1″	Maximum 258 mμ.; inflexions 280, 290 mμ.
2″	Maximum 265 mμ.; inflexion 290 mμ.
3″	Maxima 255, 261 mμ.; inflexion 275 mμ.

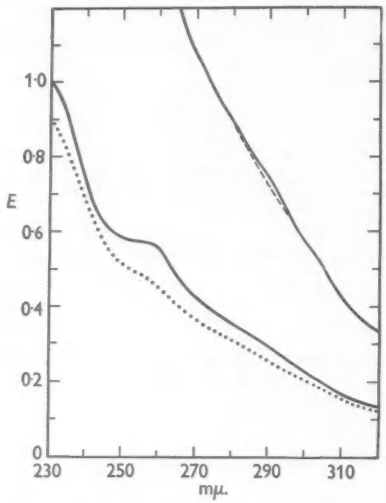


Fig. 5. Sebum unsaponifiable material (in *cyclo*hexane). ——, Sample 1, lower curve 0·04 %; upper curve is lower curve × 2·5. · · · · ·, Sample 2, 0·05 %.

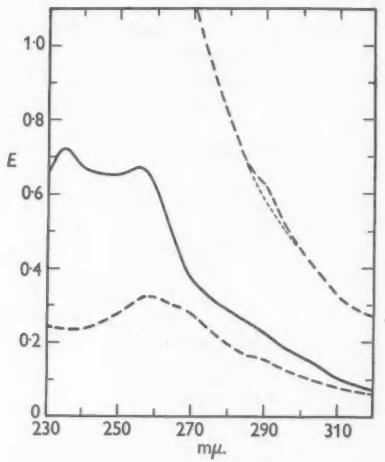


Fig. 6. Chromatographed sebum unsaponifiable material (sample 1) (in *cyclo*hexane). ——, Fraction 2, 0·002 % – – – –, fraction 3, lower curve 0·001 %, upper curve is lower curve × 4.

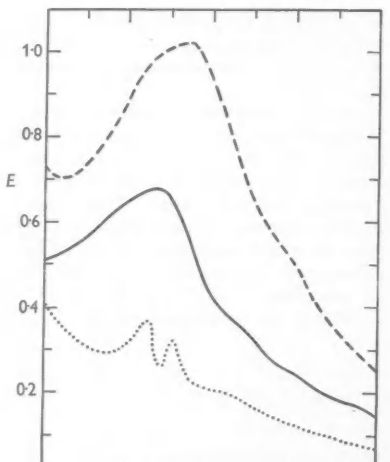


Fig. 7. Twice rechromatographed sebum unsaponifiable material (sample 1). ——, Fraction 1″; – – – –, fraction 2″; · · · · ·, fraction 3″.

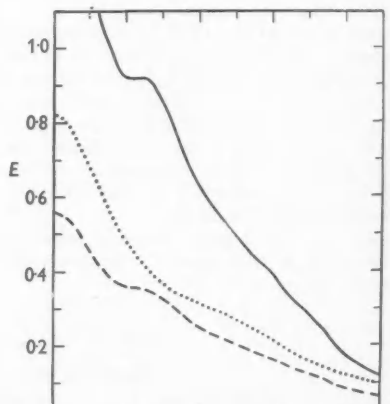


Fig. 8. Chromatographed sebum unsaponifiable material (sample 2) (in *cyclo*hexane). ——, Fraction 1, 0·095 %; – – – –, fraction 2, 0·023 %; · · · · ·, fraction 3, 0·063 %.

The unsaponifiable sample (2) showed $E^{1\%}_{1\,cm.}$ 295 mμ. 4·5; if an inflexion is present at 290 mμ. it is very faint indeed (Fig. 5). Chromatography (Table 4) was carried out on 'weakened' alumina to minimize oxidation—in case the alumina was responsible for oxidation of any tocopherol.

Only fractions 1 and 2 showed selective absorption, and this was not very pronounced (Fig. 8). It is interesting to compare the curves with those obtained from chromatography of the first sample (see Fig. 6): the maxima at 235 mμ. and in the vicinity of 260 mμ. are not so evident, neither is there an inflexion at 290 mμ. except possibly for fraction 1.

The absorption of fraction 2 is shown in Fig. 10; it is the chief fraction showing selective absorption near 290 mμ. The lower curves show correction assuming 20 and 10 % of the absorption at 295 mμ. to be contributed by α-tocopherol. It is evident from consideration of the 'corrected' curves that there may be small amounts of tocopherol, but the main absorbing entity is not tocopherol.

Table 6. *Chromatography of sebum unsaponifiable fraction*

(0·37 g., on 'weakened' alumina column 12 × 1·4 cm.)

Fraction no.	Eluant	% of original wt.	$E^{1\%}_{1\,cm.}$ 295 mμ.	Character of ultraviolet absorption spectrum
1	10% ether/p*	29·6	3·1	Inflexion 260–290 mμ.
2	10% ether/p	21·2	2·0	Weak inflexion, 260–290 mμ.
3	50% ether/p	29·6	2·9	Chiefly end absorption
4	50% ether/p	7·3	4·3	
5	Ether	4·3	7·7	
6	Ethanol	4·8	5·0	

Correlation of reducing properties and spectrophotometric data

Fraction no.	$FeCl_3$-reducing substance as 'α-tocopherol' (μg.)	$E^{1\%}_{1\,cm.}$ 295 mμ. due to 'α-tocopherol'	$E^{1\%}_{1\,cm.}$ 295 mμ. obs.	Contribution to absorption by 'α-tocopherol' (%)
1	13	0·009	3·1	0·3
2	28	0·027	2·0	1·4
3	50	0·035	2·9	1·2
4	16	0·046	4·3	1·1
5	18	0·087	7·7	1·1
6	53	0·23	5·0	4·6

* p = light petroleum.

Correlation of absorption spectra and $FeCl_3$-reducing properties of sebum unsaponifiable fractions

The sample was provided by Dr V. R. Wheatley and Prof. A. Wormall. It had been saponified in the presence of pyrogallol according to the method of Tošić & Moore (1945); hydrocarbons had been removed and the remainder assayed for $FeCl_3$-reducing substances. The value 0·86 mg. 'α-tocopherol'/g. of sample was obtained. The absorption spectrum showed a weak inflexion between 260 and 290 mμ., $E^{1\%}_{1\,cm.}$ 295 mμ. = 3·2. After chromatographic separation (Table 5), the various fractions were separately tested for the presence of $FeCl_3$-reducing substances by a modification of the Emmerie-Engel colorimetric test used by Tošić & Moore (1945).

It should be noted that the $FeCl_3$-reducing properties are spread over all the fractions; there is destruction of reducing power on chromatography, but less than 50 %. The fraction which contained the highest absolute (and relative) amount of $FeCl_3$-reducing substances was fraction 6. Assuming the reducing material to be exclusively tocopherol, the absorption contributed by the latter would be of the order of 5 % of the total. The absorption curve only shows a very faint inflexion at about 280 mμ. (see Fig. 9); correction of this curve (assuming tocopherol to be the reducing entity) eliminates some of the absorption but the inflexion is not entirely eliminated.

Rechromatography of this fraction was carried out as previously, except that the material was initially dissolved in a solution of 1 % ethanol in light petroleum (Table 7).

Table 7. *Rechromatography of richest $FeCl_3$-reducing fraction*

(0·016 g., on 'weakened' alumina column 8 × 0·9 cm.)

Fraction no.	Eluant	Character of ultraviolet absorption spectrum
1	1% ethanol/p*	Weak inflexion 250–260 mμ.
2	5% ethanol/p	Inflexion 265–290 mμ.
3	10% ethanol/p	Weak inflexion 270–290 mμ.
4	10% ethanol/p	Mainly end absorption
5	10% ethanol/p	
6	20% ethanol/p	
7	Ethanol	

* p = light petroleum.

Table 8. *Chromatography of 'sterol-like' fraction from sebum unsaponifiable matter*

(0·100 g., on 'weakened' alumina column 9 × 0·9 cm.)

Fraction no.	Eluant	% of original wt.	Character of ultraviolet absorption spectrum
1	5% ethanol/p*	9	Maximum 233 mμ.: $E^{1\%}_{1\,cm.}=132$; weak maximum 278 mμ.: $E^{1\%}_{1\,cm.}=43$
2	7·5% ethanol/p	50	Weak maximum 235 mμ.: $E^{1\%}_{1\,cm.}=83$
3	10% ethanol/p	9	Chiefly end absorption
4	20% ethanol/p	2	

* p = light petroleum.

'Sterol-like' fraction. This fraction was obtained from Dr V. R. Wheatley and Prof. A. Wormall; for the method of obtaining it see MacKenna *et al.* (1952). The spectrum of the fraction showed a weak inflexion at 265–320 mμ., $E^{1\%}_{1\,cm.}$ 280 mμ. = 27·5. It was chromatographed as shown in Table 8. The absorption of fraction 2 is shown in Fig. 11.

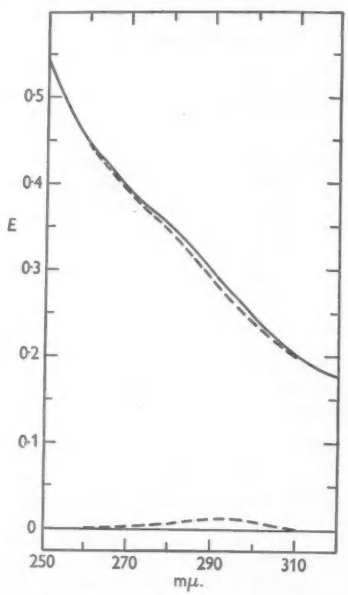


Fig. 9. Spectrum of most potent $FeCl_3$-reducing fraction and the effect of correcting for α-tocopherol as absorbing material. (In ethanol.)

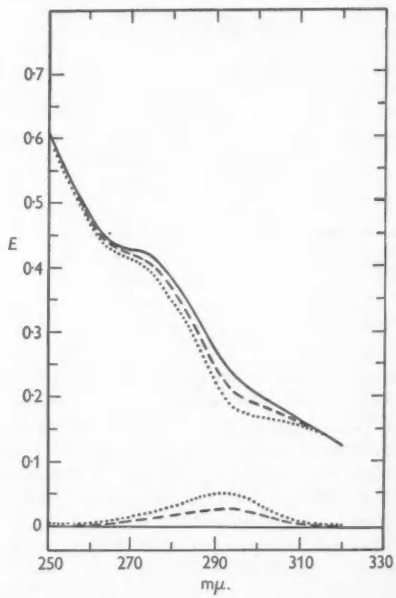


Fig. 10. Fraction from rechromatography of most potent $FeCl_3$-reducing concentrate and correction for α-tocopherol assuming 10 and 20% of the absorption at 295 mμ. to be contributed by the tocopherol.

Both fractions 1 and 2 gave a blue colour with 90% trichloroacetic acid (after Rosenheim, 1929) and with antimony trichloride in chloroform (after Carr & Price, 1926). Both these colorations showed principal absorption maxima in the vicinity of 660 mμ. when examined on the Hilger-Nutting instrument; in the Rosenheim test fraction 1 also gave a faint 600 mμ. band.

As pointed out by MacKenna *et al.* (1952) it is possible that the compound responsible for the coloration is 7α-hydroxycholesterol. 7α-Hydroxycholesterol dibenzoate (m.p. 175°) was therefore examined: a band at 660 mμ. accompanied by a weaker one at 575 mμ. was observed in the Carr-Price test.

At no stage in the work was spectrophotometric evidence obtained for the presence of provitamins D, vitamins A and K or carotene.

Chemical investigations of the various fractions obtained from chromatography of sebum unsaponifiable material, sample 2, were carried out as follows:

The hydrocarbon fraction (1) was three times recrystallized from boiling ethanol when it was possible to separate a white wax-like material, m.p. 56° (C, 85·12; H, 14·42%; mol.wt. (Rast), 560). The remainder of the fraction was taken up in acetone and treated with dry hydrogen chloride gas. After taking to dryness some crystalline material separated (m.p. 106–109°; Cl, 32·5%). This was probably a mixture of the various isomers of squalene hydrochloride (Heilbron, Kamm & Owens, 1926). The calculated Cl content of squalene hydrochloride is 33·86%.

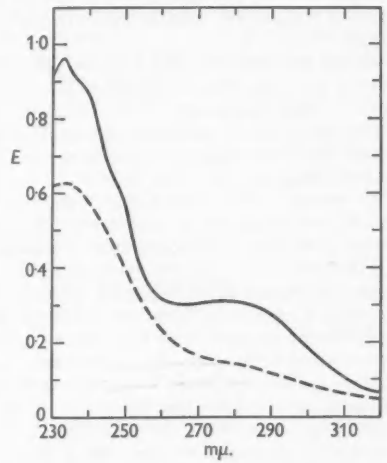


Fig. 11. Chromatography of 'sterol-like' fraction (in ethanol). ——, Fraction 1, 0·0072%; – – – –, fraction 2, 0·0075%.

The main sterol fraction (4) was twice recrystallized from methanol and shown to be cholesterol (m.p. 145°).

The strongly adsorbed fraction (7) was recrystallized from methanol when some light yellow-brown crystalline material (m.p. 79–81°) was obtained.

In these investigations the findings of MacKenna *et al.* (1950) have been confirmed.

DISCUSSION

The primary question is whether there is any vitamin E in sebum. The first sample of sebum unsaponifiable material has a spectrum with an inflexion at 290 mμ. (see Fig. 5). Correction for 'irrelevant absorption' (see p. 171) results in an estimate of 0·3% tocopherol for the particular fraction examined. This fraction after chromatographic separation yielded two fractions with inflexions at 290 mμ.: one with maxima at 235 and 255 mμ. as well, and the other with a maximum at 258 mμ. Again using the method of extrapolation for eliminating 'irrelevant absorption', the tocopherol in the original fraction is 0·1% calculated from fraction 2 and 0·06% calculated from fraction 3.

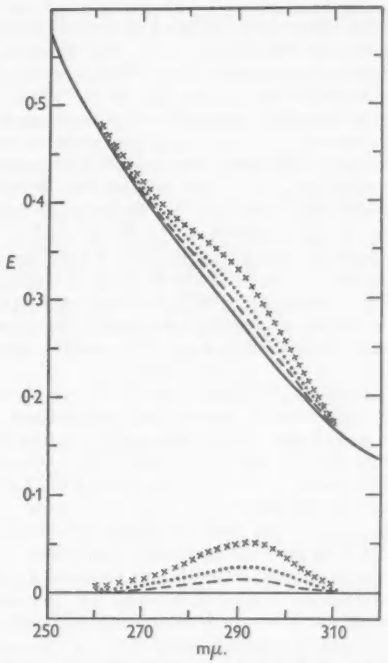


Fig. 12. Spectrum of sebum (sample 1) plus addition of 5, 10 and 20% of absorption at 295 mμ. as α-tocopherol.

These estimates are at best very approximate, yet there is an indication that there is loss of '290 mμ. material' on chromatography. It is doubtful whether the absorption at 290 mμ. is due to tocopherol or is merely part of the absorption spectrum of the 235 mμ. and/or the 255 mμ. absorbing material.

Some of the fractions obtained from the sebum itself (see fraction 2, Fig. 2, and fraction 2, Fig. 4) show faint inflexions at 290 mμ. There, too, there is other and more pronounced selective absorption—at 235 and 255 mμ. It seems certain, however, that the 290 mμ. material was not produced during the saponification process.

Assessment of tocopherol content where there is no 295 mμ. maximum is not easy. An approximate

figure for the percentage tocopherol required to produce an inflexion in a sample showing mainly end absorption has been calculated as follows: a curve showing chiefly end absorption—in this case of sebum sample 1—was plotted; then assuming 5% of the absorption at 295 mμ. to be due to tocopherol, the effect of superimposing the amount on the original curve is shown. The procedure is also carried out for the effect of 10 and 20% of the absorption at 296 mμ. estimated as tocopherol (Fig. 12).

In the case of 20% the effect on the curve is quite marked, and 10% produces a distinct inflexion. It is therefore suggested that it is a reasonable approximation to assume that if no inflexion is evident then the absorption due to tocopherol cannot exceed 5% of the total absorption observed at 295 mμ. (The values for the tocopherol absorption spectrum have been interpolated from the graphical data provided by Robeson & Baxter (1943) for α-tocopherol, $E^{1\%}_{1\,cm.}$ 292 mμ. = 73.) On this basis the maximum amount of tocopherol in any of the non-saponifiable fractions is 0·03% for the first sample and 0·02% for the second sample (expressed as percentage of sebum). The amount assessed by MacKenna *et al.* (1952) is of the order of 0·02%.

Cuthbertson, Ridgeway & Drummond (1940) have carried out the spectroscopic examination of various rat tissues; they claim that small amounts of tocopherols may easily be missed when they are insufficient (i.e. $<0{\cdot}1$%) to produce an inflexion in the ultraviolet absorption curve of the fat itself. They also found that amounts of tocopherols sufficient to give rise to distinct inflexions in the absorption curves of the crude extracts from tissues do not always reveal themselves by maxima at 290–294 mμ. Low concentrations tend to influence the curve in the vicinity of 270–280 mμ.; increasing concentration tends to shift the position of the maximum towards the region characteristic of the tocopherols. According to Cuthbertson *et al.* direct spectroscopic estimation of tocopherols in crude tissue extracts is unreliable if the proportion is much below 1%, but examination of the unsaponifiable fraction will enable quantities down to 0·1% to be determined satisfactorily. These conclusions have been drawn from experiments where known amounts of tocopherol were added to rat kidney fat and rat body fat. (These rats were fed on a vitamin E-deficient diet and the spectra of the original extracts show end absorption only.)

Regarding the detection of 0·1% in unsaponifiable material, this would correspond to 0·03% as percentage of sebum (MacKenna *et al.* (1950) quote the unsaponifiable content of sebum as 32%).

The inflexions between 260 and 300 mμ. may on this basis be indicative of small amounts of tocopherols; this assumption is best treated with caution. The fraction obtained from rechromatography of the richest $FeCl_3$-reducing material (see Fig. 10) shows a distinct inflexion between 260 and 290 mμ. Correcting this curve for tocopherol eliminates some of the selectively absorbing material, but not all of it, suggesting the presence of something other than tocopherol.

It is of interest in this connexion to recall the findings of Moore & Rajagopal (1940), who investigated the spectroscopic properties of various rat tissues, with special reference to vitamin E content. Unsaponifiable fractions of tissues were examined from: (1) vitamin E-deficient rats; (2) rats fed on a diet reinforced with synthetic α-tocopherol; (3) rats fed on a diet containing wheat-germ oil. In all the rats absorption due to unidentified substances was shown, usually by an inflexion at 280 mμ.

It should be borne in mind that the selectively absorbing material may be oxidation products of squalene. MacKenna *et al.* (1952) have detected oxidation products of squalene in three of their five principal fractions obtained from sebum unsaponifiable material.

With regard to the 'sterol-like' material, if it were 7-hydroxycholesterol, then it should be precipitated by digitonin, and according to MacKenna *et al.* (1952) it is not. The only evidence MacKenna *et al.* have in favour of the substance being a sterol is a positive Liebermann-Burchard and Rosenheim test. It has been suggested by Dr T. Moore that the compound may arise by heating cholesterol.

Kaishio & Gardner (1930) found that specimens of cholesterol heated in open test tubes at various temperatures from 160 to 200° developed a blue colour with Rosenheim's reagent, with a band near 660 mμ. and a faint subsidiary band near 610 mμ. This appears to be identical with the fractions tested. It is known that the 7 position of the sterol nucleus is the most susceptible to oxidation, and it is quite likely that Kaishio & Gardner produced 7-hydroxycholesterol by heating cholesterol. 7-Hydroxycholesterol has no absorption band at 235 or 278 mμ., but 7-ketocholesterol would show a maximum in the vicinity of 235 mμ. and it may well accompany 7-hydroxycholesterol as the next oxidation product.

SUMMARY

1. Spectrophotometric studies have been carried out on human sebum and sebum unsaponifiable fractions. The principal selectively absorbing substance shows $\lambda_{max.} = 258$ mμ. This has not been identified.

2. No vitamin A, β-carotene, vitamin K or provitamin D has been detected.

3. Vitamin E may be present in small amounts. The evidence is doubtful; if tocopherol is present it is outside the range of detection of normal spectroscopic measurement. It is estimated that the amount of tocopherol cannot exceed 0·03 % of the sebum.

4. Two fractions from a 'sterol-like' substance provided by MacKenna *et al.* (1952) have been shown to both give a blue colour with trichloroacetic acid ($\lambda_{max.}$ 660 mμ.) and with antimony trichloride in chloroform ($\lambda_{max.}$ 664 mμ.). One shows $\lambda_{max.}$ 235 mμ. and the other $\lambda_{max.}$ 278 mμ. (weak) as well.

5. Cholesterol, squalene, a hydrocarbon, m.p. 56°, and an unidentified substance, m.p. 79–81°, have been isolated.

This work has been undertaken at the request of Prof. A. Wormall of St Bartholomew's Hospital Medical College, London, and we are indebted to him for all the sebum samples used.

We are indebted to the Medical Research Council for a grant to the work of the Department. One of us (G.N.F.) held the Johnston Fellowship in the University of Liverpool.

REFERENCES

Carr, F. H. & Price, E. A. (1926). *Biochem. J.* **20**, 497.
Cuthbertson, W. F. J., Ridgeway, R. R. & Drummond, J. C. (1940). *Biochem. J.* **34**, 34.
Emmerie, A. (1940). *Rec. Trav. chim. Pays-Bas*, **59**, 246.
Evans, H. M., Emerson, O. H. & Emerson, G. A. (1936). *J. biol. Chem.* **113**, 319.
Festenstein, G. N. & Morton, R. A. (1951). *Biochem. J.* **48**, xxxix.
Heilbron, I. M. Kamm, E. D. & Owens, W. M. (1926). *J. chem. Soc.* p. 1630.
Kaishio, Y. & Gardner, J. A. (1930). *Biochem. J.* **24**, 1047.
MacKenna, R. M. B., Wheatley, V. R. & Wormall, A. (1950). *J. invest. Derm.* **15**, 33.
MacKenna, R. M. B., Wheatley, V. R. & Wormall, A. (1951). *Biochem. J.* **48**, xxxviii.
MacKenna, R. M. B., Wheatley, V. R. & Wormall, A. (1952). *Biochem. J.* **52**, 161.
Moore, T. & Rajagopal, K. R. (1940). *Biochem. J.* **34**, 335.
Robeson, C. D. & Baxter, J. G. (1943). *J. Amer. chem. Soc.* **65**, 940.
Rosenheim, O. (1929). *Biochem. J.* **23**, 47.
Tošić, J. & Moore, T. (1945). *Biochem. J.* **39**, 498.

The Reactivity of Radiomimetic Compounds

1. CROSS-LINKING OF PROTEINS

By P. ALEXANDER, M. FOX, K. A. STACEY and L. F. SMITH
Chester Beatty Research Institute of the Royal Cancer Hospital, London, S.W. 3, and Department of Inorganic and Physical Chemistry, Imperial College, London, S.W. 7

(*Received* 6 *November* 1951)

Within the last five years a number of different compounds has been shown to produce biological effects similar to those of X-rays. Radiomimetic activity has been defined by Loveless (1951) as a highly specific effect upon the resting cell producing an alteration of the genetic material which is revealed by chromosome breakage and rearrangement in subsequent divisions. Radiomimetic compounds are powerful growth inhibitors and have found limited use in the chemotherapy of malignant disease. Several have been demonstrated to be both carcinogenic and mutagenic (Haddow, 1951). Chemically, all the compounds in this class can be considered as alkylating agents (Loveless, 1951); they include sulphur mustard and nitrogen mustards, epoxides, ethyleneimines and *O*-methanesulphonyl derivatives of hydroxy compounds. Haddow, Kon & Ross (1948) demonstrated that, in the bischloroethylamine (nitrogen mustards) series, the structural condition requisite for biological activity is the presence of two halogenoalkyl groups. It has since been found that this requirement holds throughout the whole series and that, with the exception of a few isolated cases, only compounds with two alkylating groups in the molecule are active in the intact animal (Hendry, Homer, Rose & Walpole, 1951; Haddow, private communication). In tests *in vitro*, unifunctional compounds are capable of bringing about radiomimetic effects, but only at concentration 50 to 100 times those required for corresponding multifunctional compounds (see Biesele, Philips, Thiersch, Burchenal, Buckley & Stock, 1950; Loveless & Ross, 1950).

These observations led Goldacre, Loveless & Ross (1949) to propose the hypothesis that the radiomimetic compounds exert their biological action by cross-linking genetic material such as chromatid threads. This hypothesis could not be tested directly,

but has found support in the findings that mustard gas cross-links nucleic acid (Elmore, Gulland, Jordan & Taylor, 1948) and alginic acid (Deuel & Neukom, 1949) and that bisepoxides cross-link alginic acid and pectin (Deuel, 1947) and wool (Capp & Speakman, 1949; Fearnley & Speakman, 1950).

To determine if a correlation exists between biological activity and the ability to cross-link proteins, a representative selection of radiomimetic compounds and some multifunctional but inactive substances was examined.

The two proteins studied were crystalline bovine serum albumin and wool. The soluble protein was treated in aqueous solution and cross-linking was measured directly by determining the change in molecular weight by light scattering. In the case of wool, which is already extensively cross-linked by disulphide bonds, the formation of additional cross-links can be determined only after the disulphide links have been broken.

EXPERIMENTAL

Materials

Crystalline bovine serum albumin was obtained from Armour Laboratories, Lindsey St., London, E.C. 1, and found to be electrophoretically homogeneous in potassium phosphate buffer, pH 7·2, ionic strength 0·1. Root ends of Lincoln wool fibres were used after purification by successive Soxhlet extractions with ethanol and ether.

Compounds not commercially obtainable were prepared in the Organic Chemistry Department of the Chester Beatty Research Institute and kindly supplied by Mr G. M. Timmis, Dr W. C. J. Ross and Mr J. L. Everett. The dihalogen derivatives of dinitrobenzene were kindly provided by Imperial Chemical Industries Ltd. For the compounds shown in Table 2, the methods of preparation are given as follows: compound no. 1, Ward (1935); no. 3, Ross (1949); no. 7, Everett & Kon (1950). Compounds nos. 11–16 were prepared by the action of methanesulphonyl chloride on the corresponding alcohols (Timmis, 1950, 1951).

Treatment of wool fibres

Wool fibres (1 g.) were suspended in 25 ml. water, $NaHCO_3$, borate or acetate buffer (depending on the pH required) containing 3×10^{-3} moles of the reagent. If the reagent was not sufficiently soluble, the excess was present as solid. Solution of the less water-soluble compounds in aqueous acetone or ethanol did not lead to more extensive reaction and these mixed solvents were therefore not used. The reaction mixture was shaken at 40° for 1 day and then left standing for 2 days at the same temperature. The mustards and mesyloxy compounds (see Table 2) hydrolyse in water with the liberation of acid, and it is therefore difficult to maintain the pH constant. High concentration of buffer cannot be used, since all the radiomimetic alkylating compounds readily react with anions (see, for example, Ross, 1950), which compete with the proteins for the reagent. To study the reactions at different pH values a compromise had to be made between pH stability and the extent of the competing reaction; 0·4 M–0·5 M-sodium borate or acetate buffers and $NaHCO_3$ were therefore used. With $NaHCO_3$ the pH remained steady at 8·2, but with the other buffers pH changes of as much as 1 unit occurred. Epoxides hydrolyse without change in pH, and in the case of the ethyleneimines the pH change is small, so that 0·1 M buffer was sufficient.

Modified wool fibres

The amino groups of wool were acetylated by the I.G. Farben process (Alexander & Whewell, 1947), and 90% of the ϵ-NH_2 groups appeared to have reacted in this way. (This value was determined by treating the fibres with 1-fluoro-2:4-dinitrobenzene and estimating the amount of dinitrophenyllysine formed.) The carboxyl groups were esterified by refluxing wool fibres in anhydrous 0·1 N-methanolic HCl for 4 hr. (Alexander, Carter, Earland & Ford, 1951).

Cross-linking of wool fibres

Speakman (1947) showed that a wool fibre contracts in length after treatment with a solution of $NaHSO_3$ at 100° ('supercontraction'), but that this contraction was prevented if new non-disulphide cross-links were introduced. This method is not, however, completely unambiguous, since the extent of contraction depends also on the relative number of charged groups in the fibre. For example, thorough esterification of the carboxyl groups brings about a diminution in the degree of contraction (Blackburn & Lindley, 1948). This difficulty was eliminated by investigating the action of the corresponding uni- and bi-functional compounds in parallel experiments which enables effects due to esterification only and to cross-linking to be differentiated. Contraction is also diminished without cross-linking by depositing large quantities of polymers within the fibres. As some of the compounds studied are known to polymerize (Hendry *et al.* 1951), the extent of reaction was so adjusted that the increase in weight of the wool fibres was not greater than 5% (except for the halogeno-2:4-dinitro compounds, which cannot polymerize), since this amount of polymer does not affect fibre contraction. The bisulphite test may fail to detect cross-links if these are not stable to the severe conditions employed in the test, and for this reason the contraction and solubility of fibres after oxidation with peracetic acid was determined. This reagent, used at room temperature, confines its attack to the disulphide bonds and after 90% of these have been oxidized, the fibre contracts in length (Alexander, 1951) and dissolves in 3 N-aqueous ammonia leaving only the subcuticle (10% of the whole fibre). The reaction with peracetic acid is relatively mild, and, even after contraction, the fibre retains its α-keratin structure, whereas bisulphite at 100° brings about complete disorientation. Only by making these several tests and taking the precautions outlined is it felt that cross-linking can be unambiguously established and distinguished from other possible effects.

Bundles of fibres were tied at each end with cotton thread, and the distance between the tie bands measured. The fibres were then immersed in 5% (w/v) $NaHSO_3$, maintained at 100°, withdrawn after 1 hr., rinsed and dried between filter papers; the length was again measured. The contraction was expressed as percentage change in length. The contraction in peracetic acid was determined after treatment for 25 hr. at 22° in 1·6% peracetic acid prepared by the method of Greenspan (1947). The solubility of the fibres after oxidation with peracetic acid was determined by

treating a weighed bundle of fibres as above, immersing in 0·3 N-aqueous ammonia for 24 hr. at room temperature and filtering the residue on a coarse sintered-glass filter. All determinations were carried out in duplicate. Control samples of fibres treated only in buffer were tested simultaneously. Satisfactory reproducibility was obtained as long as fibres from the same batch were used. Treatment of wool fibres at pH > 9 at 40° for 3 days leads to extensive formation of lanthionine cross-links which prevent contraction in bisulphite (Speakman, 1947), or in peracetic acid (Alexander, 1951), and solubilization of the oxidized wool in dilute NH_3. For this reason it was impossible to carry out reactions at pH > 9. The small number of cross-links introduced at 40° at pH 9, though not sufficient to influence contraction, can diminish the solubility of the oxidized fibre (see Table 1), and this is therefore an extremely sensitive method of detecting cross-links.

Determination of molecular weight by light scattering

The reaction of the radiomimetic compounds with bovine serum albumin was examined at two concentrations of protein, 0·5 and 2 %, and with 10^{-2} and 2×10^{-2} M solutions of the reagents (4×10^{-2} M solution of compound no. 1) respectively in 0·1 M-sodium phosphate buffer to maintain the pH at 7. (In the experiments with epoxides no buffer was present.) The protein and reagents were carefully filtered through porcelain candles with a pore size of 0·5 μ. diameter, mixed together in sterile vessels and allowed to react at 40° for 5 days. The reaction mixture was cooled to room temperature and the light scattered at right angles measured for various dilutions made with filtered 0·1 M-phosphate buffer to maintain constant ionic strength. The intensity of the light (I) scattered by the protein was compared with that scattered by pure nitrobenzene and the results expressed as the relative scatter, $\tau = I_{\text{protein}}/I_{\text{nitrobenzene}}$. From a graph of c/τ against c (where c is the concentration of protein in g./ml.) the mol.wt. was determined from the intercept at zero concentration. This method has been used by Edsall, Edelhoch, Lontie & Morrison (1950). Their value of the molecular weight for the untreated protein was accepted as a standard and the other molecular weights determined by reference to this value. The same molecular weight was obtained for solutions of protein immediately after mixing and after keeping for 5 days at 40° indicating that no aggregation of the untreated protein occurred, as was found by Bier & Nord (1949) with egg albumin. During filtration through 0·5 μ. filters, 10–20 % of the protein of the stock 4 % solution of protein was retained by the filter. This ensured complete clarification, but made it impossible to filter a heterogeneous reaction product since the larger particles would be preferentially retained.

RESULTS

Cross-linking of soluble proteins

Comparison of Figs. 1 and 2 shows that cross-linking occurred only in protein solutions containing 2 % protein, even though in the more dilute (0·5 %) solution twice the proportion of reagent to protein was used. An increase in molecular weight, i.e. cross-linking, is indicated when the intercept on the ordinate is less than that for untreated protein. This behaviour is similar to that found in other systems; cross-linking of alginic acid by mustard, first observed by Deuel & Neukom (1949), was found to occur only in relatively concentrated solutions of the polymer. With the sodium salt of polymethacrylic acid it has also been shown that the reaction with nitrogen mustards is almost wholly intramolecular at polymer concentrations of 0·1 % or less, but that in more concentrated solutions cross-linking leading to gel formation readily takes place (Alexander, unpublished observations).

The nitrogen mustard and the bisepoxide cross-linked serum albumin while the dimethanesulphonyl compound failed to do so, although it is a very effective cross-linking agent for wool fibres (see p. 182). Propylene oxide seems to have had a degrading effect, which is being further investigated. The pronounced change in slope of the c/τ against c curve of the protein treated with both the chloroethyldimethylamine and the bischloroethylmethylamine indicates that the charge on the protein has been increased (Edsall *et al.* 1950), although a change in molecular weight was only produced by the bifunctional compound. The change in gradient is also observed in the reaction of the nitrogen mustard with a 0·5 % protein solution when no cross-linking occurred (see Fig. 1), and it can be concluded that the nitrogen mustard has reacted intramolecularly.

Epichlorhydrin and 2:4:6-tris-(ethyleneimino)-*s*-triazine (Table 2, compound no. 9) are both very effective cross-linking agents for serum albumin, and reaction with these compounds under the conditions used in the above experiments leads to rapid precipitation of the protein. By reducing the protein concentration to 0·1 % and allowing it to react with 10^{-2} M-triazine, the degree of cross-linking was restricted and the complex remained in solution. Since, however, the triazine itself polymerizes to give an insoluble product, the solution of the cross-linked protein was faintly opalescent and turbidity measurements could not be made. The polymer could not be separated by filtration since the cross-linked protein molecules were too large to pass through the fine pores, which indicates that they were of high molecular weight.

Cross-linking of wool fibres

The results obtained with twenty different compounds are given in Table 2. The corresponding treatments in buffer solution only, are given in Table 1. A surprising feature is the inability of the aliphatic nitrogen mustard (compound no. 2) and the sulphur mustard (compound no. 4) to cross-link wool fibres, although these compounds have been shown to cross-link other systems. The effect of the nitrogen mustard was examined under other conditions of pH and concentration besides those given in

Table 2, but no evidence for cross-linking was found in any of these experiments. The conditions of the test are unlikely to bring about rupture of the

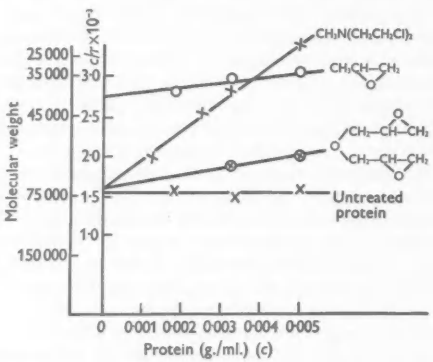


Fig. 1. Molecular weight determinations by light scattering of dilutions of a 0·5% (w/v) solution of bovine serum albumin after treatment with radiomimetic compounds (1×10^{-2} M). For details see text.

new bond, which would then not be revealed, since cross-linking was well established by the methods used for the chemically closely related compounds nos. 3, 9 and 10. To eliminate altogether the possibility that the new cross-links are broken in subsequent treatments, the contraction of fibres in aqueous phenol was examined. For the compounds mentioned above, this test also failed to reveal the presence of new cross-links. Elöd & Zahn (1944) found this method suitable for detecting cross-linkage formation and it has been used by us for examining fibres treated with all the compounds shown in Table 2. The results so obtained were in every case in accord with those given by the peracetic acid and bisulphite tests, but are not recorded since it was impossible to obtain satisfactory quantitative reproducibility and also because the rationale of the test is not yet understood. The difference in the reaction of the aliphatic nitrogen mustard (compound no. 2) with a soluble protein and wool cannot be explained. All the reactions with proteins involve a number of simultaneous and competitive reactions with the buffer solution, and profound differences between a homogeneous and a heterogeneous system are therefore not surprising.

The aromatic nitrogen mustard (compound no. 3), although capable of cross-linking wool fibres, is not nearly as effective as epichlorhydrin (no. 6) and the bisepoxide (compound no. 7). Epichlorhydrin derives its bifunctional character from its chlorine atom, which can react under mild conditions with amino groups. Alternatively, after epichlorhydrin has reacted via its epoxy group, elimination of hydrochloric acid may occur to form a new epoxy group and this could provide a second reaction centre. Although this reaction has been shown to take place under relatively mild conditions (Alexander *et al.* 1951), it is unlikely to occur at pH 7.

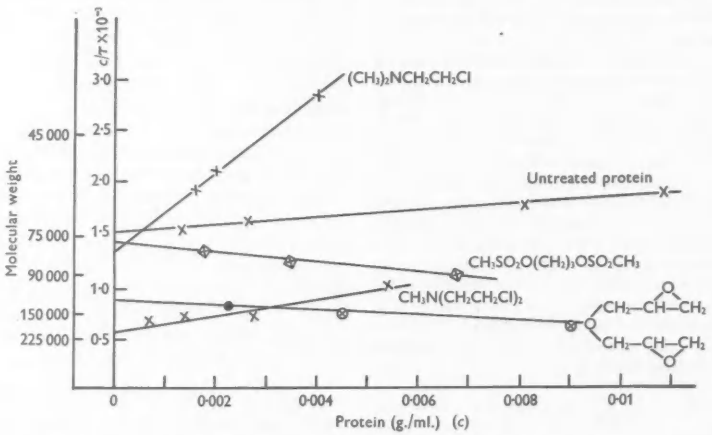


Fig. 2. Molecular weight determination by light scattering of dilutions of a 2% (w/v) solution of bovine serum albumin after treatment with radiomimetic compounds (2×10^{-2} M, except for $(CH_3)_2.N.CH_2CH_2Cl$ which was 4×10^{-2} M). For details see text.

The polyethyleneimines readily cross-link wool and compound no. 9 is outstanding. These substances were originally developed in Germany for cross-linking regenerated cellulose fibres, and in this application compounds nos. 9 and 10 found the

widest use (Alexander & Whewell, 1947). All the bifunctional mesyloxy derivatives, with the exception of compound no. 12, cross-link wool; compound no. 15 is more effective than compound no. 14, although both substances have similar chemical reactivities (Timmis, 1951). The failure of compound no. 12 to bring about cross-linking cannot be attributed to its low reactivity (Timmis, 1951) since treatment for 7 days, which is sufficient for complete hydrolysis of the compound, did not result in cross-linking.

Table 1. *Contraction and solubility of wool fibres after treatment with buffer solution for* 3 *days at* 40°

Buffer (pH)	Contraction after reaction with: Bisulphite (%)	Contraction after reaction with: Peracetic acid (%)	Percentage of oxidized fibre insoluble in ammonia
Distilled water	27·9	19·0	12
pH 2	32·6	13·4	12
pH 4	30·4	12·8	13
pH 7	30·0	12·5	26
pH 9	24·8	11·6	40
$NaHCO_3$ (pH 8·2)	32·4	10·5	15

Changes of pH seem to affect the cross-linking reaction of all the different types of compounds in the same way (compare compounds nos. 3, 7, 9 and 13). No cross-linking occurs in treatments at or below pH 4, and the reaction appears to have an optimum at pH 7. Solutions more alkaline than pH 9 could not be used since these brought about the formation of lanthionine cross-links.

Chemical considerations suggest that the amino- and carboxyl-groups in wool are most likely to react with the radiomimetic compounds. In an attempt to determine which of these are involved, a selected number of the compounds was examined with wool samples in which the carboxyl and amino groups were blocked by esterification and acetylation, respectively. Table 3 shows that with the exception of the very effective 2:4:6-tris-(ethyleneimino)-*s*-triazine (compound no. 9), none of the compounds cross-linked acetylated wool, but in nearly all cases their effectiveness was not impaired by esterification. Only the mesyloxy compound did not react with esterified fibres.

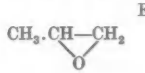
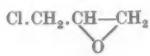
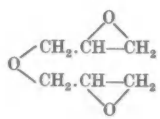
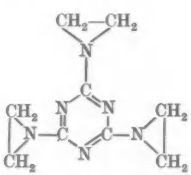
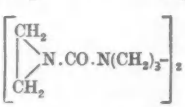
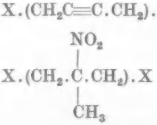
Abderhalden & Stix (1923) and Sanger (1945) demonstrated that 1-halogeno-2:4-dinitrobenzenes react with many of the reactive groups in proteins. Recently, Zahn (1951) showed that 1:5-difluoro-2:4-dinitrobenzene brought about a measure of cross-linking in wool. This observation has been confirmed and the corresponding dichloro and dibromo compounds (compounds nos. 18–20) were found to act similarly. 1-Fluoro-2:4-dinitrobenzene (compound no. 17) can bring about similar changes (i.e. diminished contraction and solubility after disulphide bond breakdown), though to a lesser extent than the bifunctional compounds, and is the only unifunctional compound to do so.

DISCUSSION

All the bifunctional radiomimetic compounds examined can cross-link proteins, though not necessarily in the same system. The results do not, however, lend support to the cross-linking hypothesis of radiomimetic activity (Goldacre *et al.* 1949), since the cross-linking tests used are unable to differentiate between biologically active and inactive multifunctional compounds. Thus, although the trisethyleneimino compound (no. 9) is both the most highly biologically active compound and one of the best cross-linking agents, the dihalogenodinitrobenzene derivatives and epichlorhydrin have no radiomimetic activity in the intact animal (Haddow, private communication), but are powerful cross-linking agents for wool and serum albumin. The lack of correlation between ability to cross-link and biological activity is best shown in the series of bifunctional mesyloxy compounds (nos. 11–16). Timmis (1951) found that the butanediol derivative (no. 14) has the highest biological activity and that this falls with decreasing chain length; introduction of an acetylenic bond (in compound no. 15) or substitution as in compound no. 16 removes all biological activity without altering significantly the chemical reactivity, but these two substances are among the most effective cross-linking agents examined. The ability of compound no. 15 to effect such powerful cross-linking may perhaps be ascribed to the linear structure of the molecule imposed by the triple bond, which prevents intramolecular reaction. This type of reaction is known to occur with the saturated compounds of this series (e.g. no. 14), and may involve ringing round on an amino group (Timmis, 1951), or reaction with adjacent groups on the same macromolecule (cf. Alexander, 1952). The failure of the ethylene glycol ester (compound no. 12) to cross-link wool is difficult to explain as in this case, also, steric factors would favour inter- rather than intramolecular reaction.

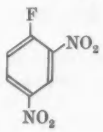
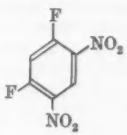
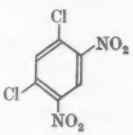
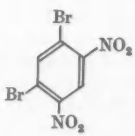
The only unifunctional compound (no. 17) which brings about changes comparable to cross-linking is one containing a 2:4-dinitrobenzene group in addition to an alkylating group. The apparent cross-linking action probably results from the strong secondary forces by which dinitrobenzene nuclei are held to one another, or to groupings already present in the wool fibre. A comparable effect occurs when wool is reduced followed by alkylation of the sulphydryl groups (Patterson, Geiger, Mizell & Harris, 1940). The strength of the wool fibre after

Table 2. *Cross-linking of wool fibres by radiomimetic and related compounds*

Reagent no.		pH of solution	Contraction after reaction with: Bisulphite (%)	Contraction after reaction with: Peracetic acid (%)	Percentage of oxidized fibre insoluble in ammonia
	Nitrogen and sulphur mustards				
1	$(CH_3)_2N.CH_2.CH_2.Cl$	5·8	32·0	14·8	—
2	$CH_3.N.(CH_2.CH_2.Cl)_2$	5·8	29·6	11·4	26
		8·2 ($NaHCO_3$)	31·2	14·5	19
3	p-$CH_3O.C_6H_4.N.(CH_2.CH_2Cl)_2$	4 (approx.)	26·4	8·8	15
		7 (approx.)	29·3	11·6	58
		8·2 (glycine-NaOH)	15·4	14·1	48
4	$S.(CH_2.CH_2.Cl)_2$	* 8·2 ($NaHCO_3$)	25·1	11·0	39
	Epoxides				
5		7 (water only)	27·2	14·7	14
6		7 (water only)	3·8	2·3	75
7		7 (water only)	0·9	1·5	78
		9	1·0	—	76
		2	25·9	—	22
	Ethyleneimines				
8		4	27·1	11·9	—
		7	24·7	15·3	13
9		4	30·7	13·9	—
		7·5	1·8	—	86
		9	2·0	—	68
10		7	12·9	6·1	—
	Methanesulphonyloxy derivatives (X is CH_3SO_2O—)				
11	$X.(CH_2)_3.CH_3$	8·2 ($NaHCO_3$)	24·5	13·1	—
12	$X.(CH_2)_2.X$	8·2 ($NaHCO_3$)	25·1	13·9	—
13	$X.(CH_2)_3.X$	4	25·6	9·2	11
		7	6·5	3·5	76
		8·2 ($NaHCO_3$)	5·0	2·4	88
14	$X.(CH_2)_4.X$	8·2 ($NaHCO_3$)	7·7	11·7	62
15	$X.(CH_2C{\equiv}C.CH_2).X$	8·2 ($NaHCO_3$)	0·0	13·4	74
16		8·2 ($NaHCO_3$)	1·5	13·2	63

* Applied from 50% ethanol.

Table 2 (*cont.*)

Reagent no.	Halogeno-2:4-dinitrobenzenes*	pH of solution	Contraction after reaction with: Bisulphite (%)	Contraction after reaction with: Peracetic acid (%)	Percentage of oxidized fibre insoluble in ammonia
17		8·2 ($NaHCO_3$)	17·8	14·0	48·5
18		8·2 ($NaHCO_3$)	5·6	9·0	80·5
19		8·2 ($NaHCO_3$)	12·8	11·3	73·0
20		8·2 ($NaHCO_3$)	3·0	11·4	68·6

* Applied from 75% ethanol.

treatment is much less when a short-chain unifunctional alkylating agent such as methyl bromide is used, than when long-chain groups (e.g. heptyl), capable of interaction by secondary forces with other groups, are introduced. It is interesting in this connexion that *N*-2:4-dinitrophenyl-ethyleneimine is one of the few unifunctional compounds which has a biological activity comparable to that of multifunctional compounds (Hendry *et al.* 1951), and it may be that one of the chemically reactive groups in radiomimetic compounds can be replaced by a group capable of powerful interaction by secondary forces.

Table 3. *Cross-linking of modified wool fibres by radiomimetic compounds*

Reagent (numbered as in Table 2)	Contraction after reaction with bisulphite: Acetylated fibres (%)	Contraction after reaction with bisulphite: Esterified fibres (%)
None	28·2	25·0
No. 7	31·2	9·2
No. 6	28·5	1·7
No. 9	6·4	5·5
No. 13	27·3	26·5

The results with chemically modified wool fibres (see Table 3) show that the amino groups, but not the carboxyl groups, play an important part in the cross-linking reaction and the cross-links may be formed between pairs of amino groups. Chemical considerations suggest that all radiomimetic compounds react by a carbonium ion mechanism and that they can react only with amino groups which are unionized and carboxyl groups which are dissociated (Ross, 1949). This is the case in model systems such as polymethacrylic acid and the polymer of ethyleneimine (Alexander, unpublished observations); in these, cross-linking occurs only with the ionized form of the former and the undissociated form of the latter polymer.

The optimum pH range found for cross-linking wool (7–8) would suggest reaction with carboxyl rather than with amino groups, since the majority of the latter are in the ionized and therefore unreactive state. The contrary result obtained would indicate that the chemical reactivity of these groups may not

be correctly represented as $—NH_3^+$ and $—COO^-$ and that in fact they behave in chemical reaction as $—NH_2$ and —COOH groups near the isoelectric point, where they neutralize one another. This suggestion is supported by the observation (Rutherford, Patterson & Harris, 1940) that isoelectric proteins can be esterified with diazomethane, which in simple systems only reacts with unionized carboxyl groups.

SUMMARY

1. To examine the hypothesis that the radiomimetic alkylating agents function by a cross-linking mechanism, the action of a representative group of these compounds has been studied with serum albumin in solution and with wool.

2. It was shown from molecular weight determinations using light scattering that the serum albumin is cross-linked in a 2% (w/v) solution by the nitrogen mustards, bisepoxides and tris-(ethyleneimino)triazine.

3. Examination of the physical properties of wool fibres after reaction with twenty radiomimetic and related compounds showed that cross-linking can be brought about by most of these substances.

4. There appears to be no correlation between the biological activity of a compound and its effectiveness as cross-linking agent in the systems examined.

5. Experiments with acetylated and esterified wool fibres indicate that the cross-links involve a reaction with amino groups.

The authors would like to express their appreciation to Prof. A. Haddow for his help and encouragement.

This investigation has been supported by a special grant from the Anna Fuller Fund and by grants to the Royal Cancer Hospital and Chester Beatty Research Institute from the British Empire Cancer Campaign, the Jane Coffin Childs Memorial Fund for Medical Research and the National Cancer Institute of the National Institutes of Health, U.S. Public Health Service.

REFERENCES

Abderhalden, E. & Stix, W. (1923). *Hoppe-Seyl. Z.* **129**, 143.
Alexander, P. (1951). *Bull. N.Y. Acad. Sci.* **53**, 653.
Alexander, P. (1952). *Nature, Lond.*, **169**, 226.
Alexander, P., Carter, D., Earland, C. & Ford, O. E. (1951). *Biochem. J.* **48**, 629.
Alexander, P. & Whewell, C. S. (1947). *British Intelligence Objectives Sub-committee Report*, no. 1472. London: H.M. Stationery Office.
Bier, M. & Nord, F. F. (1949). *Proc. nat. Acad. Sci., Wash.*, **35**, 17.
Biesele, J. J., Philips, F. S., Thiersch, J. B., Burchenal, J. H., Buckley, S. M. & Stock, C. C. (1950). *Nature, Lond.*, **166**, 1112.
Blackburn, S. & Lindley, H. (1948). *J. Soc. Dy. Col., Bradford*, **64**, 305.
Capp, C. W. & Speakman, J. B. (1949). *J. Soc. Dy. Col., Bradford*, **65**, 402.
Deuel, H. (1947). *Helv. chim. Acta*, **30**, 1523.
Deuel, H. & Neukom, H. (1949). *J. Polymer Sci.* **4**, 755.
Edsall, J. T., Edelhoch, H., Lontie, R. & Morrison, P. R. (1950). *J. Amer. chem. Soc.* **72**, 4641.
Elmore, D. T., Gulland, J. M., Jordan, D. O. & Taylor, H. F. W. (1948). *Biochem. J.* **42**, 308.
Elöd, E. & Zahn, H. (1944). *Kolloidzschr.* **108**, 94.
Everett, J. L. & Kon, G. A. R. (1950). *J. chem. Soc.* p. 3131.
Fearnley, C. & Speakman, J. B. (1950) *Nature, Lond.*, **166**, 743.
Goldacre, R. J., Loveless, A. & Ross, W. C. J. (1949). *Nature, Lond.*, **163**, 667.
Greenspan, F. P. (1947). *Industr. Engng Chem.* **39**, 847.
Haddow, A. (1951). *Practitioner*, **167**, 36.
Haddow, A., Kon, G. A. R. & Ross, W. C. J. (1948). *Nature, Lond.*, **167**, 338.
Hendry, J. A., Homer, R. F., Rose, F. L. & Walpole, A. C. (1951). *British J. Pharmacol.* **6**, 235, 35.
Loveless, A. (1951). *Nature, Lond.*, **167**, 338.
Loveless, A. & Ross, W. C. J. (1950). *Nature, Lond.*, **166**, 1116.
Patterson, W. I., Geiger, W. B., Mizell, L. R. & Harris, M. (1940). *Bur. Stand. J. Res., Wash.*, **25**, 451.
Ross, W. C. J. (1949). *J. chem. Soc.* p. 183.
Ross, W. C. J. (1950). *J. chem. Soc.* p. 2257.
Rutherford, H. A., Patterson, W. I. & Harris, M. (1940). *Bur. Stand. J. Res., Wash.*, **25**, 451.
Sanger, F. (1945). *Biochem. J.* **39**, 507.
Speakman, J. B. (1947). *J. Text. Inst., Manchr*, **38**, T102.
Timmis, G. M. (1950). *British Empire Cancer Campaign, Annual Report*, p. 58.
Timmis, G. M. (1951). *12th Int. Congr. Pure and Applied Chem. Abstr.*, p. 334.
Ward, K. N. (1935). *J. Amer. chem. Soc.* **57**, 914.
Zahn, H. (1951). *Kolloidzschr.* **121**, 39.

The Effect of Fluoride on the Succinic Oxidase System

By E. C. SLATER and W. D. BONNER, jun.
Molteno Institute, University of Cambridge

(*Received* 18 *October* 1951)

Although it has been known since the work of Battelli & Stern (1910) that the intracellular oxidation of succinate is inhibited by fluoride, the mechanism of this inhibition is little understood. The intracellular oxidation of succinate by oxygen is catalysed by a complex of enzymes, the succinic oxidase system. Our present knowledge of this system is summarized by the reaction scheme succinate → succinic dehydrogenase → cytochrome *b* → intermediary factor → cytochrome *c* → cytochrome *a* → cytochrome a_3 → O_2, where the arrows describe the pathway taken by two hydrogen atoms of succinate (or the electrons derived therefrom).

Potter & Schneider (1942) refer to unpublished work of O'Kane, who found that fluoride inhibits succinic dehydrogenase competitively with respect to succinate. This suggests that the locus of the fluoride action is the reaction between succinate and its dehydrogenase. Dufait & Massart (1939) and Borei (1945), on the other hand, found that the complete succinic oxidase system was more susceptible to fluoride than the dehydrogenase component. This seemed to indicate that some other component of the system, as well as the dehydrogenase, was attacked by fluoride. From other experiments, Borei concluded that cytochrome *c* was this component, but Slater (1949*a*, *b*) considered this unlikely and suggested that the intermediary factor, acting between cytochrome *b* and cytochrome *c*, might be the sensitive component. Subsequent experiments (Slater, 1950*a*) showed, however, that the dihydrocozymase oxidase system, which includes both cytochrome *c* and the same intermediary factor, was not affected by fluoride (see also Potter, 1939). It follows, therefore, that neither cytochrome *c* nor the intermediary factor can be the point of attack of fluoride on the succinic oxidase system.

Further studies are clearly necessary to resolve these discrepancies. Bonner's (1951) recent finding that phosphate is required for the inhibitory action of fluoride made this particularly desirable, since it indicates a reaction between phosphate and some component of the enzyme system. It is now established that phosphate is esterified during the aerobic oxidation of succinate by mitochondrial preparations (e.g. Cross, Taggart, Covo & Green, 1949), although phosphate is not necessary for the oxidation itself (Keilin & Hartree, 1949; Bonner, 1951, 1952). It seems possible that the reaction between the enzyme system and phosphate which is necessary for the inhibition by fluoride might be related to some reaction between phosphate and a component of the enzyme system, which must occur if the oxidation of succinate is accompanied by esterification of phosphate. The fact that high concentrations of fluoride are customarily employed in studies of oxidative phosphorylation was an additional reason for studying its mode of action.

A preliminary account of this work has already appeared (Slater & Bonner, 1951).

METHODS

Keilin-Hartree heart-muscle preparation was the preparation containing very little inorganic phosphate, made as described by Bonner (1952). Concentrations of heart-muscle preparation are expressed as mg. fat-free dry wt./ml.

Enzyme activities were all measured at pH 7·2–7·4.

Succinic oxidase system. Two methods were employed.

(i) Manometric method. After temperature equilibration, the reaction was commenced by addition of succinate from a dangling tube and the O_2 uptake was followed. Except where otherwise stated, cytochrome *c* was not added. The total fluid volume was 3·3 ml.

(ii) Spectrophotometric method. This depends upon measurement of the rate of formation of fumarate by determining the rate of increase of optical density at 230 mμ. This procedure has been used by Chance (1952) for the succinic oxidase system and by Racker (1950) for measuring fumarase activity. The method is only valid for the succinic oxidase system if the preparation is essentially free of fumarase. This was found to be the case with the Keilin & Hartree preparation, but not with the phosphorylating preparation used by Slater (1950*b*). A 1 cm. silica cell contained succinate and any other additions in a total volume of 2·8 ml. The reference cell contained no succinate. At zero time, 0·2 ml. of heart-muscle preparation, suitably diluted (usually 50-fold), was added to each cell and the optical density followed (in a Hilger 'Uvispek' spectrophotometer) as a function of time. The temperature of the solution was measured. The activity was expressed as increase of optical density at 230 mμ./min., corrected to 20°, applying the temperature coefficient found by Slater (1950*a*).

Succinic dehydrogenase. Two methods were employed.

(i) Manometric method. This was as previously described (Slater, 1949*f*), with the exception that the dangling tube contained the diluted heart-muscle preparation, not the succinate. This was to prevent inactivation, during temperature equilibration, of the succinic dehydrogenase by the

cyanide (0·01 M), which Tsou (1951) has found occurs only in the absence of succinate. This method measures the rate of reduction of methylene blue by succinate, which may be a function not only of the succinic dehydrogenase concentration, but also of the cytochrome *b* (Slater, 1949*f*; Tsou, 1951).

(ii) Spectrophotometric method. The rate of reduction of $K_3Fe(CN)_6$ (0·001 M) was measured in the presence of sufficient KCN (0·01 M) to inhibit cytochrome oxidase, by following the rate of decrease of optical density at 400 mμ. A 1 cm. cell contained the succinate, cyanide and ferricyanide in a total volume of 2·8 ml. The reference cell contained water only. At zero time, 0·2 ml. of heart-muscle preparation, suitably diluted (usually tenfold), was added to both cells and the optical density at 400 mμ. followed as a function of time. The rate of change of optical density decreased somewhat during the measurement. The average rate between 1 and 5 min. after addition of the enzyme was used for calculation of the activities. The temperature of the solution was measured and the rate corrected to 20°, assuming the same temperature coefficient as found for the complete succinic oxidase system by Slater (1950*a*). It may not be valid to use the same temperature correction, but the range of temperature in the one experiment was small, so that any error caused by uncertainty in the correction will be negligible. It is not known what components of the succinic oxidase system are involved in the reduction of $K_3Fe(CN)_6$ (cf. Slater, 1949*b*), but it is probable that succinic dehydrogenase is the most important.

Reagents

Sodium succinate. This was standardized manometrically.

Phosphate buffer, pH 7·24. In order to ensure that this contained no pyrophosphate, which is a strong competitive inhibitor of succinic dehydrogenase (Leloir & Dixon, 1937; Tsou, 1951), the buffer was prepared in the following manner. Orthophosphoric acid (49 g.) was dissolved in approximately 500 ml. water. The solution was boiled for several hours, then diluted to about 900 ml., brought to pH 7·24 (glass electrode) with concentrated NaOH and diluted to 1000 ml. The phosphate concentration, measured by the molybdenum blue method, was 0·447 M.

EXPERIMENTS

Mechanism of the action of fluoride

The action of fluoride, in the presence of phosphate, on that portion of the succinic oxidase system responsible for the reduction of potassium ferricyanide, is shown in Fig. 1. It is clear that the inhibition by fluoride is a rapid reaction. The same degree of inhibition was found irrespective of whether fluoride was added to the enzyme 10 min. after or before the succinate. This is in disagreement with Borei (1945), who found that prior treatment of the enzyme preparation with succinate gave protection against fluoride.

Also in contrast with Borei (1945), it was found that the inhibition was completely reversed by dialysis against phosphate buffer. The reversibility of the inhibition is shown also by the experiment described in Fig. 2. Concentrated heart-muscle preparation (1 ml.) was treated with 1·0 M-NaF (0·1 ml.) and 0·15 M-phosphate, pH 7·3 (0·9 ml.), for 1 hr. at 22°. The concentration of fluoride during the treatment was 0·05 M and that of phosphate, 0·067 M. The activity of the complete succinic oxidase system was measured after dilution of this mixture 187-fold; the final fluoride concentration was then $2·7 \times 10^{-4}$ M. A control was treated in the same way, without fluoride, except for the addition of $2·7 \times 10^{-4}$ M in the test for enzyme activity. The

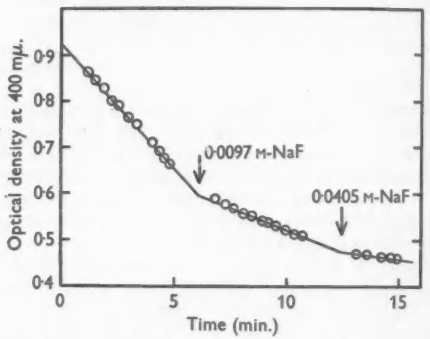


Fig. 1. Inhibition by fluoride of succinic dehydrogenase, measured spectrophotometrically at 20°. Phosphate, 0·14 M; succinate, 0·026 M; $K_3Fe(CN)_6$, 0·001 M; KCN, 0·01 M; heart-muscle preparation, 0·25 mg./ml.

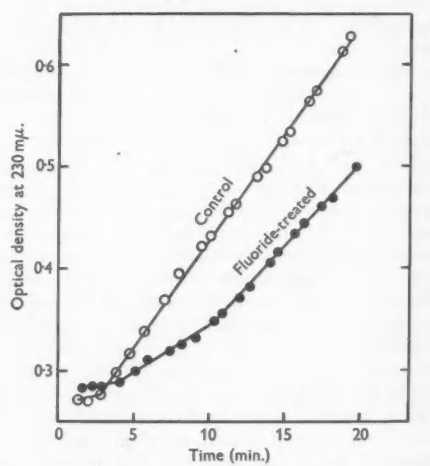


Fig. 2. Reversibility of fluoride inhibition of succinic oxidase system. See text for treatment of 'control' and 'fluoride-treated'. Activity of succinic oxidase system measured spectrophotometrically at 21·3°; phosphate, 0·12 M; succinate, 0·027 M; heart-muscle preparation, 0·062 mg./ml.

succinic oxidase activities of the two preparations are shown in Fig. 2. It can be seen that, after an initial lag, the fluoride-treated preparation has nearly the same activity as the control. Thus, even prolonged treatment with 0·05 M-fluoride, which is sufficient to inhibit completely the enzyme (see below), has practically no irreversible effect.

The effect of phosphate on the degree of inhibition by fluoride is shown in Table 1. As already found by Bonner (1951), phosphate is necessary for any appreciable inhibition by fluoride; with increasing phosphate, the inhibition by fluoride is increased.

Arsenate can replace phosphate, but is less effective (Table 2).

creasing succinate concentration, becoming zero at infinite succinate concentration.

Table 1 shows that, in the absence of fluoride, phosphate is itself somewhat inhibitory (cf. Keilin & Hartree, 1949; Slater 1949*c*). This inhibition has been further studied in Fig. 4. Unlike the experiments summarized in Tables 1 and 3, the complete succinic oxidase system was not studied in

Table 1. *Effect of phosphate on the degree of inhibition of the succinic oxidase system by fluoride*

(Activity of succinic oxidase system measured manometrically at 25°. All flasks contained succinate, 0·021 M; borax-boric acid buffer, pH 7·3, 0·1 M; histidine, pH 7·3, $8{\cdot}1 \times 10^{-3}$ M; heart-muscle preparation, 0·36 mg./ml.; NaF (0·01 M) and phosphate where shown.)

$$V = v_p\{1 + 0{\cdot}023(1 + p/0{\cdot}1)\} \text{ (see text, p. 191).}$$

[Phosphate] (M)	Succinic oxidase system (μl. O_2/hr.) No NaF (v_p)	+NaF (v)	Inhibition by NaF (%)	V	V/v
0	349	315	9·6	357	1·13
0·03	319	198	38·0	327	1·65
0·06	274	108	60·6	284	2·62
0·10	252	92·5	63·2	264	2·86
0·15	235	57	76	248	4·35
0·20	209	46	78	224	4·89

Table 2. *Comparison of effect of phosphate and arsenate on the inhibition of the succinic oxidase system by fluoride*

(Activity of succinic oxidase system measured spectrophotometrically at 20–22°. Succinate, 0·027 M; heart-muscle preparation, 0·031 mg./ml. Activity expressed as increase of optical density at 230 mμ./min.)

[Phosphate] (M)	[Arsenate] (M)	[NaF] (M)	Succinic oxidase system	Inhibition by NaF (%)
0·12	0	0	0·0178	72
0·12	0	0·0097	0·0050	
0	0·12	0	0·0190	29
0	0·12	0·0097	0·0132	

Table 3. *Effect of different concentrations of fluoride on the degree of inhibition of the succinic oxidase system*

(Activity of succinic oxidase system measured manometrically at 25°. All flasks contained succinate, 0·021 M; borax-boric acid buffer, pH 7·3, 0·1 M; histidine, pH 7·3, $8{\cdot}1 \times 10^{-3}$ M; heart-muscle preparation, 0·36 mg./ml. Phosphate (0·1 M) and fluoride where shown.)

$$V = v_f\{1 + 0{\cdot}023(1 + f/0{\cdot}02)\} \text{ (see p. 192),}$$
$$v' = 331\ v/226 \text{ (see p. 192).}$$

[Fluoride] (M)	Succinic oxidase system (μl. O_2/hr.) No phosphate (v_f)	+phosphate (v)	Inhibition (%)	V	v'	V/v'
0	331	226	31·9	340	331	1·02
0·002	296	185	37·5	302	271	1·11
0·005	293	114	61·1	300	167	1·80
0·008	276	84	69·6	294	123	2·30
0·012	326	72	77·9	338	105	3·22
0·015	277	49	82·3	288	72	4·01

The effect of various concentrations of fluoride, at a fixed concentration of phosphate (0·1 M), is shown in Table 3.

In Fig. 3 the effect of fluoride on the enzyme system in a phosphate medium at different substrate concentrations is shown, plotted according to the method of Lineweaver & Burk (1934). The results clearly show that the inhibition is competitive, i.e. the degree of inhibition decreases with increasing succinate concentration.

this experiment, but only that portion of it responsible for the reduction of potassium ferricyanide, which is probably mainly succinic dehydrogenase. The reason for this change was that it seemed likely from previous work (see Slater, 1949*c*, Fig. 2) that phosphate had a much stronger action on the complete system than on the dehydrogenase, whereas it was clear from the fact that fluoride was a competitive

inhibitor that it acted exclusively on succinic dehydrogenase (see below). Fig. 4 shows that phosphate is a competitive inhibitor of succinic dehydrogenase.

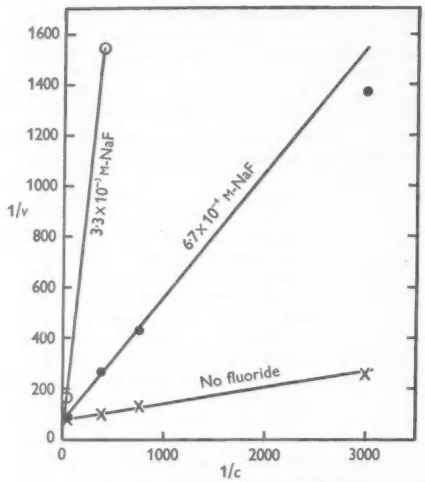


Fig. 3. Competitive nature of fluoride inhibition of succinic oxidase system. Activity measured spectrophotometrically at 19·1–21·2° and calculated to 20°; phosphate, 0·12 M; heart-muscle preparation, 0·06 mg./ml. c = molar concentration of succinate, v = increase/min. of optical density at 230 mμ.

In the absence of phosphate, fluoride is a weak competitive inhibitor of succinic dehydrogenase.

Phosphate and fluoride are not the only inorganic anions which inhibit succinic dehydrogenase. Chloride and nitrate are considerably more inhibitory than phosphate, but this inhibition is largely non-competitive; the degree of inhibition is less at high than at low substrate concentrations, but is still high at infinite substrate concentrations.

The addition of magnesium had no effect on the inhibition by fluoride and phosphate (Table 4). In this respect, the inhibition of succinic dehydrogenase by fluoride differs from that of enolase, which inhibition also requires phosphate (Warburg & Christian, 1942). Manganese also had no effect.

Component of succinic oxidase system affected by fluoride

Added cytochrome c did not affect the degree of inhibition by fluoride (Table 5), showing that it is not the susceptible component of the system. This experiment does not agree with a similar one by Borei (1945).

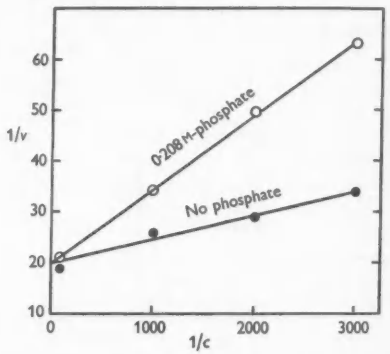


Fig. 4. Competitive inhibition of succinic dehydrogenase by phosphate. Activity measured spectrophotometrically at 24·2–25·2° and corrected to 20°; histidine, pH 7·3, $8{\cdot}1 \times 10^{-3}$ M; heart-muscle preparation, 0·16 mg./ml. pH of all reagents adjusted to pH 7·3 (glass electrode). c = molar concentration of succinate, v = increase of optical density at 400 mμ./min.

Borei's finding that the succinic dehydrogenase activity, as customarily measured, is considerably less sensitive to fluoride than the succinic oxidase system is confirmed by the experiment summarized in Table 6, in which the effect of a wide range of methylene blue concentrations (resulting in a 2·5 range of rate of O_2 uptake) is shown. It is seen that with increasing activity of the system in the absence of fluoride, the percentage inhibition by fluoride increases, but even at the highest concentration of methylene blue used, when the

Table 4. *Effect of magnesium and manganese on the inhibition by fluoride of the succinic oxidase system*

(Activity of succinic oxidase system measured manometrically at 38° (Exp. 1) or 25° (Exp. 2). Phosphate, 0·13 M; succinate, 0·024 M; heart-muscle preparation, 0·26 mg./ml. (Exp. 1) or 0·36 mg./ml. (Exp. 2).)

[Mg] (M)	[Mn] (M)	[NaF] (M)	Succinic oxidase system (μl. O_2/hr.)	Inhibition by NaF (%)
		Exp. 1		
0	0	0	474	57·6
0	0	0·01	200	
0·001	0	0	480	58·4
0·001	0	0·01	200	
		Exp. 2		
0	0	0	224	74·1
0	0	0·01	58	
0	0·001	0	235	76
0	0·001	0·01	56	

Table 5. *Effect of cytochrome* c *on the degree of inhibition of the succinic oxidase system by fluoride*

(Activity of succinic oxidase system measured manometrically at 38°. Phosphate, 0·13 M; succinate, 0·024 M; heart-muscle preparation, 0·28 mg./ml.)

[Cyt. *c*] ($M \times 10^5$)	[NaF] (M)	Succinic oxidase system (μl. O_2/hr.)	Inhibition by NaF (%)
0	0	371	70·4
0	0·01	110	
6	0	430	71·2
6	0·01	124	

O_2 uptake of the control approached that found in the measurement of the activity of the complete succinic oxidase system, the percentage inhibition was far below that found with the complete system.

The direct comparison of O_2 uptakes in the measurements of the succinic dehydrogenase and the complete succinic oxidase systems is, however, not valid; the former should be halved. This is because the final reaction with oxygen in the succinic dehydrogenase measurement is with leuco-methylene blue and the product of this oxidation is H_2O_2, thus

$$\text{succinate} + \text{meth. blue} \rightarrow \text{fumarate} + \text{leuco-meth. blue}$$
$$\text{leuco-meth. blue} + O_2 \rightarrow \text{meth. blue} + H_2O_2$$
$$\text{i.e. succinate} + O_2 \rightarrow \text{fumarate} + H_2O_2$$

The formation of H_2O_2 in this reaction was demonstrated by the addition of a large amount of pure catalase, which halved the O_2 uptake (Fig. 5). Heart-muscle preparation contains a little catalase, but this would be only very slightly active in the presence of 0·009 M-cyanide, which is used in the estimation of succinic dehydrogenase. The amount of catalase added in the experiment shown in Fig. 5 was such that considerable activity remained even in the presence of this concentration of cyanide. In the measurement of the succinic oxidase system, O_2 reacts with cytochrome oxidase and is reduced to water, not H_2O_2. The overall reaction is, therefore,

$$\text{succinate} + \tfrac{1}{2}O_2 \rightarrow \text{fumarate} + H_2O.$$

Thus the succinate equivalent of the O_2 consumed in the dehydrogenase test is only half that of the same amount of O_2 in the measurement of the succinic oxidase system.

Even at the highest level of methylene blue used in Table 6, the succinic dehydrogenase activity in the absence of fluoride is, then, much less than the activity of the succinic oxidase system. Thus, the succinic dehydrogenase is working at far less than its full capacity in the dehydrogenase test, which seemed a possible explanation of why it was less susceptible to fluoride. This possibility is confirmed mathematically in the next section. The experimental data are found, in fact, to fit very closely the equation derived on the assumption that the reaction between succinic dehydrogenase and its substrate is the only point of attack of fluoride.

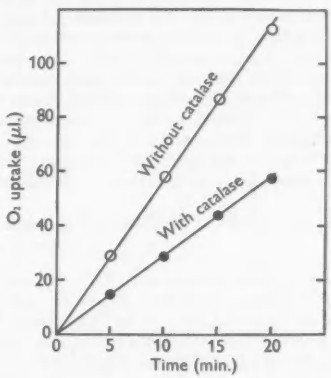


Fig. 5. Effect of catalase on O_2 uptake in manometric estimation of succinic dehydrogenase. Phosphate, 0·13 M; succinate, 0·026 M; methylene blue, $9{\cdot}2 \times 10^{-4}$ M; KCN, $9{\cdot}2 \times 10^{-3}$ M; heart-muscle preparation, 0·21 mg./ml.; catalase, 0·6 mg./ml.

Thus the relative insensitivity of the dehydrogenase compared with the succinic oxidase system is only apparent and not real, and is due to the unsatisfactory nature of the measurement of dehydrogenase activity.

Malonate, the well known competitive inhibitor of succinic dehydrogenase, had previously been found by Slater (1949*e*) to inhibit the O_2 uptake in the measurement of the succinic oxidase system more than in the dehydrogenase measurement. Undoubtedly, the explanation is the same as that given for fluoride.

The fact that succinic dehydrogenase is working far below its full capacity when it reacts with methylene blue causes difficulty only when a reversible competitive inhibitor of the

Table 6. *Inhibition of succinic dehydrogenase and of the complete succinic oxidase system by fluoride*

(Both activities measured manometrically at 38°. Phosphate, 0·13 M; succinate, 0·024 M; fluoride, 0·0091 M; heart-muscle preparation, 0·21 mg./ml.)

$V = v_p(1 + 95\ K_m)$ (see p. 192),
$K_m = 4{\cdot}8 \times 10^{-4}$ with succinic oxidase system, $3{\cdot}6 \times 10^{-7}\ v_p$ with succinic dehydrogenase (see p. 192).

Activity	[Methylene blue] ($M \times 10^4$)	Activity (μl. O_2/hr.) No NaF (v_p)	Activity (μl. O_2/hr.) +NaF (v)	Inhibition by NaF (%)	V	V/v
Succinic dehydrogenase	1·5	208	159	23·6	209	1·31
	3·0	267	193	27·7	269	1·39
	9·1	361	241	33·2	365	1·51
	30	453	291	36·0	460	1·58
Succinic oxidase system	.	663	268	59·6	690	2·58

dehydrogenase is being studied. The degree of inhibition by compounds which act by irreversibly removing a fraction of the enzyme, e.g. sulphydryl-combining reagents (Slater, 1949*d*) will be independent of the activity.

Effect of fluoride on the succinic oxidase system in a phosphorylating preparation

The experiments described above were all carried out with the Keilin & Hartree heart-muscle preparation, which, although it oxidizes succinate very rapidly, is unable to couple this oxidation with the synthesis of adenosinetriphosphate (ATP). It was of interest to see if fluoride had the same effect on a phosphorylating as on a non-phosphorylating preparation, since the former must contain enzyme systems linked with the phosphorylating reaction absent from the latter preparation, and it appeared possible that these additional systems might provide an oxidative pathway not susceptible to fluoride. The experiment summarized in Table 7 shows, however, that the succinic oxidase system in a phosphorylating mitochondrial preparation was inhibited to the same extent as that in the non-phosphorylating Keilin & Hartree preparation. Similarly, inhibition of the phosphorylation by the addition of dinitrophenol to or the omission of the adenylic system from, the mitochondrial preparation did not appreciably affect the degree of inhibition by fluoride.

Table 7. *Inhibition of succinic oxidase system by fluoride in both phosphorylating and non-phosphorylating systems*

(1·82 mg. pig-heart mitochondria (*M*); 1·91 mg. horse-heart Keilin-Hartree (*K-H*) preparation. Phosphate, 0·0348 *M*; succinate, 0·0267 M; cytochrome *c*, $5{\cdot}8\times10^{-5}$ M in all flasks. Those provided with 'adenylic system etc.' contained, in addition, adenylic acid, $1{\cdot}6\times10^{-3}$ M; ADP, $0{\cdot}63\times10^{-3}$ M; ATP, $0{\cdot}29\times10^{-3}$ M; glucose, 0·083 M; $MgCl_2$, 0·047 M and hexokinase. Dinitrophenol (DNP), 5×10^{-4} M, and NaF, 0·04 M where stated. Total volume, 3 ml. Time of exp. 30 min. Temperature, 25° with *M*; 38° with *K-H* preparation. ΔHMP (hexosemonophosphate) measured as described by Slater (1950*b*, 1951).)

Preparation	Adenylic system, etc.	DNP	NaF	O_2 uptake (μg. atoms)	ΔHMP (μmoles)	P/O	Inhibition of O_2 uptake by NaF (%)
M	−	−	−	9·51	.	.	62
M	−	−	+	3·58	.	.	
M	+	−	−	9·91	10·5	1·03	59
M	+	−	+	4·07	4·54	1·11	
M	+	+	−	9·56	0·04	0	54
M	+	+	+	4·44	0	0	
K-H	−	−	−	24·0	.	.	58
K-H	−	−	+	10·0	.	.	

CALCULATIONS

Inhibition constants

Fluoride (F), phosphate (P), but especially the two together, compete with succinate (S) for succinic dehydrogenase (E). Thus we have

$$\mathrm{E}+\mathrm{S}\underset{k_2}{\overset{k_1}{\rightleftharpoons}}\mathrm{ES}, \qquad (1)$$

$$\mathrm{ES}\xrightarrow{k'_3}\text{products}+\mathrm{E}, \qquad (2)$$

$$\mathrm{E}+\mathrm{P}\underset{k_5}{\overset{k_4}{\rightleftharpoons}}\mathrm{EP}, \qquad (3)$$

$$\mathrm{E}+\mathrm{F}\underset{k_7}{\overset{k_6}{\rightleftharpoons}}\mathrm{EF}, \qquad (4)$$

$$\mathrm{EP}+\mathrm{F}\underset{k_9}{\overset{k_8}{\rightleftharpoons}}\mathrm{EPF}\underset{k_{10}}{\overset{k_{11}}{\rightleftharpoons}}\mathrm{EF}+\mathrm{P}. \qquad (5)$$

$k_1, k_2, \ldots, k_{11}$ are true rate constants, with the exception of k'_3; k'_3 is not a true rate constant, but includes concentrations of reactants of the individual hydrogen-transferring reactions, whose sum is reaction 2. The back reaction of Eqn. (2) has been ignored, since initial rates of reaction when the fumarate concentration is low have been used throughout this study. Fig. 5, for example, shows no decrease of the rate of reaction due to fumarate production in the first 15 min.

Accepting this mechanism, it is possible to calculate from the experimental data the inhibition constants $K_P=k_5/k_4$; $K_F=k_7/k_6$; $K_{PF}=k_9/k_8$ and $K_{FF}=k_{11}/k_{10}$. The velocity (v) of the oxidation of succinate to fumarate is given by the expression (see Appendix, p. 195)

$$v=V/\{1+(K_m/s)\,(fp/K_PK_{PF}+p/K_P+f/K_F+1)\}, \qquad (10)$$

where s, f and p are concentrations of succinate, fluoride and phosphate, $K_m=(k_2+k'_3)/k_1$ is the Michaelis constant in the absence of inhibitors and V is the velocity at infinite succinate concentration.

The Michaelis constant in the presence of inhibitor (K'_m) equals $K_m(fp/K_PK_{PF}+p/K_P+f/K_F+1)$.

K_P can be calculated from the data of Fig. 4. The straight line intercepts the x-axis at a distance equal to $-1/K'_m$ from the origin. In this way, the following values were found ($f=0$)

p	$K'_m\times10^4$
0	2·3
0·208	7·25

i.e. $2{\cdot}3\times10^{-4}=K_m$,

$7{\cdot}25\times10^{-4}=K_m(1+0{\cdot}208/K_P)$.

By difference,

$$4{\cdot}95\times10^{-4}=0{\cdot}208\,K_m/K_P=0{\cdot}208\times2{\cdot}3\times10^{-4}/K_P,$$

i.e. $K_P=0{\cdot}1$.

In a similar way, K_P was found to be 0·02.

In the experiments used for the following calculations, the complete succinic oxidase system was studied, not just that portion responsible for the reduction of ferricyanide. The K_m for the complete system, which is considerably different from the value given above (see p. 194), can be calculated from the data of Fig. 3. In the absence of fluoride,

$$K'_m = K_m + pK_m/K_P.$$

Substituting the value of K'_m found ($1{\cdot}11 \times 10^{-3}$), of p (0·123) and K_P found above, $K_m = 4{\cdot}8 \times 10^{-4}$.

K_{PF} can be calculated if $K_m/K_P K_{PF}$ is known. This expression can be determined from the effect of different concentration of fluoride on the Michaelis constant (Fig. 3). This experiment was carried out with the same preparation as was used to determine K_m.

Fig. 3 yields the following values of K'_m

p	f	$K'_m \times 10^3$
0·123	0	1·11
0·123	$0{\cdot}67 \times 10^{-3}$	7·40

i.e. $$1{\cdot}11 \times 10^{-3} = K_m(1 + 0{\cdot}123/K_P)$$

$$7{\cdot}40 \times 10^{-3} = K_m(1 + 0{\cdot}123/K_P + 0{\cdot}67 \times 10^{-3}/K_P + 8{\cdot}25 \times 10^{-5}/K_P K_{PF}).$$

By difference,

$$6{\cdot}29 \times 10^{-3} = K_m(0{\cdot}67 \times 10^{-3}/K_P + 8{\cdot}25 \times 10^{-5}/K_P K_{PF}),$$

i.e.

$$6{\cdot}29 \times 10^{-3} = 0{\cdot}67 \times 10^{-3}\, K_m/K_P + 8{\cdot}25 \times 10^{-5}\, K_m/K_P K_{PF}$$
$$= 0{\cdot}02 \times 10^{-3} + 8{\cdot}25 \times 10^{-5}\, K_m/K_P K_{PF},$$

and $$K_m/K_P K_{PF} = 76.$$

Substituting the values of K_m and K_P found above,

$$K_{PF} = 6{\cdot}7 \times 10^{-5}.$$

Since $K_P K_{PF} = K_F K_{FF}$ (Eqn. (14), Appendix),

$$K_{FF} = K_P K_{PF}/K_F = 3{\cdot}3 \times 10^{-4}.$$

The value of the expression $K_m/K_P K_{PF}$ can also be calculated from the data of Tables 1 and 3. The values obtained have not been used in the above calculations for two reasons. In the first place, the experiments summarized in Tables 1 and 3 were carried out with a preparation different from that used above. Secondly, there are side reactions which complicate the calculations and require certain assumptions, the validity of which is uncertain. Nevertheless, these assumptions do not invalidate the comparison of the data of Table 1 with that of Table 3, both of which were obtained with the same enzyme preparation, and the calculations provide certain information not available from the other experiments.

Equation (11) (see Appendix) gives the relation between V, the velocity at infinite succinate concentration and v, the observed velocity.

$$V/v = 1 + (K_m/s)(1 + p/K_P + f/K_F + fp/K_P K_{PF}). \quad (11)$$

In the absence of fluoride, the velocity, v_p, is given by the expression

$$V/v_p = 1 + (K_m/s)(1 + p/K_P). \quad (11a)$$

Substituting the values for K_m and K_P, found above, and the concentration of succinate in Table 1,

$$V/v_p = 1 + 0{\cdot}023(1 + p/0{\cdot}1). \quad (11b)$$

When $p = 0$ and 0·2 respectively, $V/v_p = 1{\cdot}023$ and 1·069, i.e. the ratio of the activities of succinic dehydrogenase when the phosphate concentration is zero and 0·2 M respectively is $1{\cdot}023/1{\cdot}069 = 0{\cdot}96$. Actually, the second column of Table 1 shows that this ratio is $209/349 = 0{\cdot}60$. It is clear, then, that phosphate has an effect on the complete succinic oxidase system additional to that on succinic dehydrogenase and this must be taken into account when using the figures of Table 1 to calculate the effect of phosphate on the degree of inhibition by fluoride. It should be noted that yet another

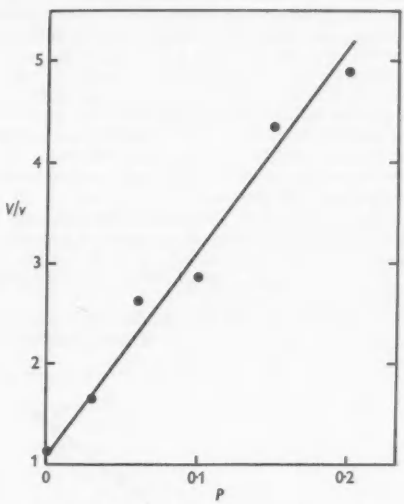


Fig. 6. Data of Table 1 graphed.
p = phosphate concentration (M).

effect of phosphate on the succinic oxidase system, namely a stimulation of the succinic oxidase system (Keilin & Hartree, 1949), is eliminated in these experiments by the addition of histidine. Bonner (1951) has found that histidine replaces phosphate in this respect, so that in its presence phosphate has no stimulatory effect.

Equation (11) may be rewritten

$$V/v = 1 + (K_m/s)(1 + f/K_F) + p(K_m/s)(1/K_P + f/K_P K_{PF}).$$

Thus, if V/v is plotted against p, a straight line should be obtained with intercept on the y-axis equal to

$$1 + (K_m/s)(1 + f/K_F)$$

and slope $(K_m/s)(1/K_P + f/K_P K_{PF})$ (cf. Burton, 1951). The data of Table 1 have been treated in this way in Fig. 6. V, the velocity at infinite concentration of succinate, has been calculated separately for each concentration of phosphate from the values of v_p in the second column of Table 1 by Eqn. (11b).

Fig. 6 shows no trend away from a straight line, although the individual points deviate somewhat from this line. The slope equals 20, i.e. $(K_m/s)(1/K_P + f/K_P K_{PF}) = 20$. Substituting for K_m, K_P, s and f, we find $K_m/K_P K_{PF} = 42$.

The experiment summarized in Table 3 can be treated similarly. In the absence of phosphate, but the presence of fluoride, the velocity of the reaction, v_f, is given by the expression

$$V/v_f = 1 + (K_m/s)(1 + f/K_F).$$

Substituting for K_m, K_P and s,

$$V/v_f = 1 + 0{\cdot}023\ (1 + f/0{\cdot}02). \qquad (11c)$$

Equation (11) may be re-written

$$V/v = 1 + (K_m/s)\,(1 + p/K_P) + f(K_m/s)\,(1/K_F + p/K_P K_{PF}),$$

and if V/v is plotted against f, a straight line with intercept $1 + (K_m/s)\,(1 + p/K_P)$ and slope $(K_m/s)\,(1/K_F + p/K_P K_{PF})$ should be obtained. In Table 3, V is calculated from v_f by Eqn. (11c). A correction must be applied to the values of v in the third column of this table to allow for the inhibition by phosphate in the absence of fluoride. It is apparent from the above that this inhibition is not due to the slight inhibition of succinic dehydrogenase, but must be due to the second effect on the complete succinic oxidase system. It is assumed that this is superimposed on the effect being studied. It cannot be influenced by the addition of fluoride, which acts only on succinic dehydrogenase. The value of the correction is given by the ratio of v_f and v in the absence of fluoride, namely 331/226.

In Fig. 7, the plot of V/v' against f fits a straight line closely, although this line does not meet the y-axis at the predicted point. The slope of the straight line is 200. Thus $(K_m/s)\,(1/K_F + p/K_P K_{PF})$ equals 200 and, by substitution, $K_m/K_P K_{PF} = 42$.

Thus the value of $K_m/K_P K_{PF}$ calculated from the experiment in which fluoride was varied at fixed phosphate concentration agrees closely with that calculated from the experiment in which phosphate was varied at fixed fluoride concentration.

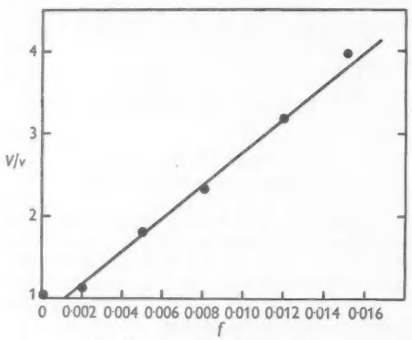


Fig. 7. Data of Table 3 graphed.
f = fluoride concentration (M).

Relative inhibition of succinic oxidase system and of succinic dehydrogenase

Equation (12) (see Appendix) shows that V/v is a linear function of V, i.e. the degree of inhibition will increase with increasing activity of the control. The data of Table 6 have been plotted in this way in Fig. 8, taking account of the fact that the values for V given in Table 6 should be divided by two when the dehydrogenase activity is measured. V has been calculated from Eqn. (11a) (see p. 191). Putting $s = 0{\cdot}024$, $p = 0{\cdot}13$ and $K_P = 0{\cdot}1$, we find, $V/v_p = 1 + 95K_m$. The value of K_m was taken as $4{\cdot}8 \times 10^{-4}$ with the succinic oxidase system (see p. 191) and

$$4{\cdot}8 \times 10^{-4} \times v_p/2 \times 663 = 3{\cdot}6 \times 10^{-7}\ v_p$$

in the dehydrogenase experiments, since it was assumed in the light of the findings below, that K_m is approximately proportional to k_3', which is proportional to V. The factor 2 is introduced for the same reason as above.

The data fit a straight line very closely.

Calculation of the rate constants of the uninhibited reaction

It is possible to calculate from Fig. 8 and some of the previous calculations, the rate constants of the uninhibited reaction.

It is shown in the Appendix that when $V/v = 1$, V equals $-k_2 e$. Thus from the extrapolation shown in Fig. 8, $-k_2 e = -48$, i.e. $k_2 e = 48\ \mu$l. O_2/hr./3·3 ml. $= 3{\cdot}6 \times 10^{-7}$ moles succinate/l./sec.

$V = k_3' e = 690\ \mu$l. O_2/hr./3·3 ml. $= 5{\cdot}15 \times 10^{-6}$ moles/l./sec.

The Michaelis constant,

$$K_m = (k_2 + k_3')/k_1 = 4{\cdot}8 \times 10^{-4},$$

i.e.

$$(k_2 e + k_3' e)/k_1 e = 4{\cdot}8 \times 10^{-4},$$

$$k_1 e = (k_2 e + k_3' e)/4{\cdot}8 \times 10^{-4}$$
$$= 5{\cdot}51 \times 10^{-6}/4{\cdot}8 \times 10^{-4}$$
$$= 1{\cdot}15 \times 10^{-2}\ \text{sec.}^{-1}.$$

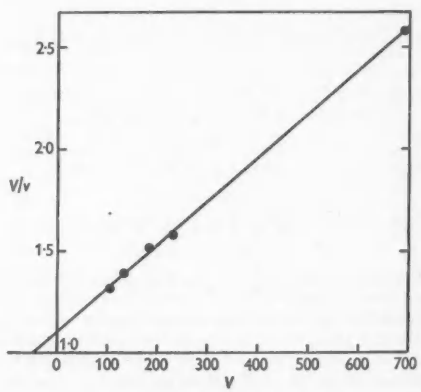


Fig. 8. Data of Table 6 graphed.

For the calculation of the actual rate constants, the enzyme concentration is required. There is no way of determining this at present. However, the above values are useful for determining the ratios of the constants, which are discussed below (see p. 194). It should be noted that the constants refer to 38°, although in the derivation of k_1, the value of K_m at 20° has been used. If the K_m is different at 38°, an error will be introduced into the calculation of k_1, but this will not be very great.

Some idea of the order of magnitude of the actual rate constants can be obtained if it is assumed that the concentration of succinic dehydrogenase in the heart-muscle preparation is of the same order of magnitude as that of cytochrome c. The fact that all the cytochrome components are present in the heart-muscle preparation in approximately the same concentration (Slater, 1949f; Chance, 1952) gives some justification for this assumption. The concentration of cytochrome c, calculated from the fat-free

dry weight and the data of Slater (1949*f*), was $0{\cdot}16 \times 10^{-6}$M in the experiment summarized in Table 6. This gives the following values: k_1, 7×10^4 l. mole^{-1} sec.$^{-1}$; k_2, 2 sec.$^{-1}$; k'_3, 32 sec.$^{-1}$. These values are probably within a factor of 10 of the true values, but should not be taken as more accurate than this.

DISCUSSION

Mechanism of action of fluoride

It has been shown that fluoride and phosphate separately have weak inhibitory effects on succinic dehydrogenase, competing with succinate for the enzyme. Fluoride and phosphate together, however, inhibit much more strongly. The fact that the inhibition constant calculated from an experiment in which phosphate was varied at fixed fluoride concentration agrees closely with that obtained when fluoride was varied at fixed phosphate concentration supports the mechanism proposed on p. 190. Experiments have not been carried out at different pH's, so it is not known which phosphate ion is inhibitory or whether it is the fluoride ion or the free acid which combines with the enzyme. Since, however, fluoride is almost exclusively in the form of fluoride ion at pH 7·3 (less than 0·01% in the form of hydrogen fluoride, Borei, 1945), it is very probable that it is the ion which is involved. The straight-line relationship shown in Figs. 6 and 7 indicate that one phosphate ion and one fluoride are involved in the inhibition. This is of interest, since inhibitions by fluoride often involve two fluoride ions, e.g. that of enolase (Warburg & Christian, 1942) and of the oxidation of quinol by cytochrome *c* and cytochrome oxidase (Borei, 1945).

The data of Table 6 and Fig. 8 clearly show that fluoride acts on the succinic oxidase system solely by virtue of its attack on succinic dehydrogenase. These experiments, therefore, give no support to Borei's (1945) view that cytochrome *c* is the fluoride-sensitive component of respiratory systems. Table 5 demonstrates this directly. Similarly, it follows that neither the cytochrome oxidase reaction nor the reaction between the endogenous cytochrome *c* in the heart-muscle preparation and cytochrome *c* oxidase are affected by fluoride. In this respect, endogenous cytochrome *c* differs from added soluble cytochrome *c*, since Borei found that the oxidation of quinol by soluble cytochrome *c* and cytochrome *c* oxidase was inhibited by fluoride and this inhibition was competitive with respect to cytochrome *c*. Borei's experiment shows that fluoride affects the reaction between soluble cytochrome *c* and the oxidase. Borei considered that this inhibition was due to a reaction between fluoride and cytochrome *c*, but since there is no other evidence for this, Slater (1949*a*) suggested that the action of fluoride was on the oxidase, similar to an effect of phosphate. It is interesting that Borei found that phosphate increased the inhibition by fluoride of this oxidation.

Succinic dehydrogenase resembles enolase in that phosphate is required for the inhibition by fluoride. This raises the question whether the dehydrogenase contains, like enolase, magnesium or possibly another metal. Such suggestions have been made previously. Massart (1939), on the basis of the inhibition by fluoride, suggested that the enzyme was a manganese compound. Because the addition of calcium increased the succinic oxidase activity of homogenates, Potter & Schneider (1942) believed that calcium was a component of the system and suggested that fluoride combined with the calcium. Later, however, it was shown (Swingle, Axelrod & Elvehjem, 1942; Keilin & Hartree, 1949) that the activation by calcium had another explanation, and there is no reason to believe that calcium is a component of the system. The lack of effect of magnesium or manganese on the degree of inhibition of succinic dehydrogenase by fluoride is in contrast with the behaviour of enolase. It cannot be excluded that the dehydrogenase is a magnesium or manganese compound but, if this is the case, the metal must be more firmly bound than in any known magnesium or manganese enzyme, since the exhaustive washing of the heart muscle would be expected to remove a considerable proportion of the metal from such enzymes.

Effect of fluoride on intracellular respiration

Fluoride in comparison with other inhibitors is not a very powerful inhibitor of succinic dehydrogenase, even in the presence of phosphate. However, if the succinate concentration is not that commonly employed when it is used as substrate in enzyme experiments but is equal to that found in cells, it becomes a powerful inhibitor. Since the oxidation of succinate is an important step in respiratory metabolism, the effect of fluoride on the respiration of intact cells (literature reviewed by Borei, 1945) might be due to its effect on succinic dehydrogenase. This would be consistent with the fact that the inhibition is reduced by any influence that increases the cells' turnover of substrate.

Enzyme kinetics

Michaelis & Menten (1913), in proposing the enzyme-substrate theory of enzyme action, which may be formulated

$$\mathrm{E} + \mathrm{S} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{ES},$$

$$\mathrm{ES} \xrightarrow{k_3} \text{products},$$

assumed that k_1 and k_2 were large compared with k_3, so that the concentration of ES was kept at a value dictated by the equilibrium of the first reaction.

Using this assumption, they derived the well known formula relating enzyme activity to substrate concentration. This relationship could, in fact, be expressed by the value of a constant, now known as the Michaelis constant (K_m), which equalled the substrate concentration at which the activity was half that obtained with infinite substrate concentration. According to Michaelis & Menten's equation, $K_m = k_2/k_1$ = the dissociation constant of the enzyme-substrate compound.

Briggs & Haldane (1925) showed that the relationship between enzyme activity and substrate concentration had the same form, if Michaelis & Menten's simplifying assumption was not made. They derived, from steady-state kinetics, the same formula as Michaelis & Menten, but

$$K_m = (k_2 + k_3)/k_1,$$

which becomes k_2/k_1 only if k_2 is large compared with k_3. Thus, in general, although it may be considered a kinetic dissociation constant when ES is reacting, K_m need not be the same as the dissociation constant of the compound in the absence of further reaction. The difference between the Michaelis and dissociation constants has also been commented upon by Hearon (1949).

Chance's (1943, 1949) measurements of the actual rate constants of horse-radish peroxidase provided the first quantitative evidence in support of the essential features of the Michaelis-Menten theory. At the same time, they showed that for this enzyme the simplifying assumption of the theory did not apply, since k_2 is in fact very small compared with k_3.

The present study, by less direct methods, has shown that, in the case of succinic dehydrogenase also, k_2 is much smaller than k_3. Thus, for both these enzymes, K_m bears no relation to the dissociation constant of the enzyme-substrate compound. The actual magnitude of the difference in the case of succinic dehydrogenase is shown by the following figures; k_2/k_1 = dissociation constant of the compound $ES \rightleftharpoons E + S$, in the absence of further reaction of ES (i.e. in the absence of hydrogen acceptor) $= 3 \times 10^{-5}$; Michaelis constant $= 4{\cdot}8 \times 10^{-4}$.

Since K_m depends on the value of k_3', which is affected by the concentration of the enzyme systems responsible for the transfer of hydrogen atoms from the ES compound to the hydrogen acceptor, there is no reason to believe that K_m will be the same for different enzyme preparations, even under otherwise identical conditions. It would be expected, however, that the dissociation constant (k_2/k_1) of succinic dehydrogenase would be a constant property of this single enzyme molecule.

The rate constants which have been calculated are based on the assumption that the Michaelis theory applies to this enzyme. The existence of competitive inhibitors, such as malonate, oxalacetate, pyrophosphate, fluoride and phosphate, makes this extremely likely, but the rate constants must eventually be determined by direct measurement, when methods are available.

Oxidative phosphorylation

In order to explain the phosphorylation of adenosinemonophosphate (AMP) or adenosinediphosphate (ADP) which accompanies the oxidation of succinate, one must assume some reaction between phosphate and the succinic oxidase system. Also to be taken into account is the fact that phosphate is not necessary for the oxidation (Keilin & Hartree, 1949; Bonner, 1951). The inhibition by phosphate alone indicates some reaction between phosphate and succinic dehydrogenase, but the reaction is non-specific and the affinity very low. In the presence of fluoride, however, phosphate has a much greater affinity for the enzyme. The possibility exists that in a phosphorylating system some other component might act like fluoride and help to bind the phosphate firmly, followed by other reactions leading to the phosphorylation of AMP or ADP. There is, as yet, no evidence to support this suggestion.

SUMMARY

1. Spectrophotometric methods for measuring the activities of the succinic oxidase system and succinic dehydrogenase are described.

2. In the manometric method of determining succinic dehydrogenase, hydrogen peroxide is an end product. Thus oxygen uptakes obtained by this method should be halved to make them comparable with activities of the complete succinic oxidase system, which yields water as the end product.

3. Fluoride and phosphate separately have only very slight effects on succinic dehydrogenase, revealed only at low substrate concentrations.

4. The inhibition by fluoride and phosphate, acting together, is much greater. It is a rapid reaction and is largely reversed by dilution.

5. All three inhibitions are completely competitive with respect to succinate. One molecule of phosphate and of fluoride react with each enzyme molecule.

6. It is concluded that succinic dehydrogenase is the only component of the succinic oxidase system susceptible to fluoride, despite the fact that succinic dehydrogenase, as customarily measured, is inhibited to a smaller degree than the complete system.

7. The reasons for the smaller inhibition in the latter case are (*a*) succinic dehydrogenase is not working at its full activity in the test, because the concentration of the hydrogen acceptor limits the rate of the reaction; (*b*) the simplifying assumptions

of the Michaelis-Menten theory do not apply to succinic dehydrogenase.

8. Arsenate behaves like phosphate but to a lesser degree.

9. Manganese and magnesium did not affect the inhibition by fluoride and phosphate.

10. Fluoride had the same inhibitory action on a phosphorylating mitochondrial preparation as on a non-phosphorylating preparation.

11. Inhibitory constants, Michaelis constants and rate constants of the uninhibited reaction have been calculated.

12. The relation between the Michaelis constant and dissociation constant of the enzyme-substrate compound is discussed.

We wish to thank Prof. D. Keilin, F.R.S., for his interest and advice in the investigation. One of us (E.C.S.) acknowledges with thanks receipt of an Australian National University Research Fellowship during part of this investigation and a personal grant from the Agricultural Research Council during the remaining part. One of us (W.D.B.) is indebted to the U.S. Public Health Service for a Fellowship during the course of the investigation.

REFERENCES

Bonner, W. D., jun. (1951). *Biochem. J.* **49**, viii.
Bonner, W. D., jun. (1952). *Biochem. J.* (To be submitted).
Borei, H. (1945). *Ark. Kemi Min. Geol.* **20 A**, no. 8.
Botelli, F. & Stern, L. (1910). *Biochem. Z.* **30**, 172.
Briggs, G. E. & Haldane, J. B. S. (1925). *Biochem. J.* **19**, 338.
Burton, K. (1951). *Biochem. J.* **48**, 458.
Chance, B. (1943). *J. biol. Chem.* **151**, 553.
Chance, B. (1949). *Arch. Biochem.* **22**, 224.
Chance, B. (1952). *Nature, Lond.*, **169**, 215.
Cross, R. J., Taggart, J. V., Covo, G. A. & Green, D. E. (1949). *J. biol. Chem.* **177**, 655.
Dufait, R. & Massart, L. (1939). *Enzymologia*, **7**, 337.
Hearon, J. Z. (1949). *Bull. math. Biophys.* **11**, 29.
Keilin, D. & Hartree, E. F. (1949). *Biochem. J.* **44**, 205.
Leloir, L. F. & Dixon, M. (1937). *Enzymologia*, **2**, 81.
Lineweaver, D. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.
Massart, L. (1939). *Hoppe-Seyl. Z.* **258**, 190.
Michaelis, L. & Menten, M. L. (1913). *Biochem. Z.* **49**, 333.
Potter, V. R. (1939). *Nature, Lond.*, **143**, 475.
Potter, V. R. & Schneider, W. C. (1942). *J. biol. Chem.* **142**, 543.
Racker, E. (1950). *Biochim. Biophys. Acta*, **4**, 211.
Slater, E. C. (1949*a*). *Biochem. J.* **44**, 305.
Slater, E. C. (1949*b*). *Biochem. J.* **45**, 14.
Slater, E. C. (1949*c*). *Biochem. J.* **45**, 8.
Slater, E. C. (1949*d*). *Biochem. J.* **45**, 130.
Slater, E. C. (1949*e*). Unpublished experiments.
Slater, E. C. (1949*f*). *Biochem. J.* **45**, 1.
Slater, E. C. (1950*a*). *Biochem. J.* **46**, 484.
Slater, E. C. (1950*b*). *Nature, Lond.*, **166**, 982.
Slater, E. C. (1951). *Biochem. J.* **50**, vii.
Slater, E. C. & Bonner, W. D., jun. (1951). *Biochem. J.* **49**, 1.
Swingle, K. F., Axelrod, A. E. & Elvehjem, C. A. (1942). *J. biol. Chem.* **145**, 581.
Tsou, C. L. (1951). *Biochem. J.* **49**, 512.
Warburg, O. & Christian, W. (1942). *Biochem. Z.* **310**, 384.

APPENDIX

Derivation of kinetic equations

The proposed mechanism (see p. 190) is

$$\underset{(e-a-b-c-d)}{\mathrm{E}} + \underset{(s)}{\mathrm{S}} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \underset{(a)}{\mathrm{ES}}, \quad (1)$$

$$\underset{(a)}{\mathrm{ES}} \xrightarrow{k_3'} \text{products} + \mathrm{E}, \quad (2)$$

$$\underset{(e-a-b-c-d)}{\mathrm{E}} + \underset{(p)}{\mathrm{P}} \underset{k_5}{\overset{k_4}{\rightleftharpoons}} \underset{(b)}{\mathrm{EP}}, \quad (3)$$

$$\underset{(e-a-b-c-d)}{\mathrm{E}} + \underset{(f)}{\mathrm{F}} \underset{k_7}{\overset{k_6}{\rightleftharpoons}} \underset{(c)}{\mathrm{EF}}, \quad (4)$$

$$\underset{(b)}{\mathrm{EP}} + \underset{(f)}{\mathrm{F}} \underset{k_9}{\overset{k_8}{\rightleftharpoons}} \underset{(d)}{\mathrm{EPF}} \underset{k_{10}}{\overset{k_{11}}{\rightleftharpoons}} \underset{(c)}{\mathrm{EF}} + \underset{(p)}{\mathrm{P}}. \quad (5)$$

The concentrations of the reactants are shown in brackets; e is the concentration of total enzyme $(\mathrm{E}+\mathrm{ES}+\mathrm{EP}+\mathrm{EF}+\mathrm{EPF})$.

Put $(k_2+k_3')/k_1=K_m$; $k_5/k_4=K_\mathrm{P}$; $k_7/k_6=K_\mathrm{F}$; $k_9/k_8=K_\mathrm{PF}$; $k_{11}/k_{10}=K_\mathrm{FP}$.

In the steady state,

$$da/dt=k_1 s(e-a-b-c-d)-(k_2+k_3')\,a=0,$$

therefore
$$(e-a-b-c-d)=(k_2+k_3')\,a/k_1 s=aK_m/s. \quad (6)$$

The concentrations of EP, EF and EPF will be governed by the equilibrium of reactions (3), (4) and (5). Thus

$$b/p(e-a-b-c-d)=1/K_\mathrm{P},$$

i.e.
$$b=(p/K_\mathrm{P})\,aK_m/s, \quad (7)$$

$$c/f(e-a-b-c-d)=1/K_\mathrm{F},$$

i.e.
$$c=(f/K_\mathrm{F})\,aK_m/s, \quad (8)$$

$$d/fb=1/K_\mathrm{PF},$$

i.e. $$d = fb/K_{FF} = (fp/K_P K_{FF})\, aK_m/s. \quad (9)$$

Substituting in (6) the values for b, c and d obtained in Eqns. (7)–(9), we find

$$a = e/\{1 + (K_m/s)\,(1 + p/K_P + f/K_F + fp/K_P K_{FF})\}.$$

The rate of the reaction,

$$v = \mathrm{d}s/\mathrm{d}t = k_3' a,$$

i.e.

$$v = k_3' e/\{1 + (K_m/s)\,(1 + p/K_P + f/K_F + fp/K_P K_{FF})\}.$$

When $s = \infty$, $v = V = k_3' e$,

i.e.

$$v = V/\{1 + (K_m/s)\,(1 + p/K_P + f/K_F + fp/K_P K_{FF})\}, \quad (10)$$

or $$V/v = 1 + (K_m/s)\,(1 + p/K_P + f/K_F + fp/K_P K_{FF}). \quad (11)$$

Expressing K_m as rate constants,

$$V/v = 1 + (k_2 + k_3') \times (1 + p/K_P + f/K_F + fp/K_P K_{FF})/k_1 s = 1 + (k_2/k_1 s)\,(1 + p/K_P + f/K_F + fp/K_P K_{FF}) + (V/k_1 se)\,(1 + p/K_P + f/K_F + fp/K_P K_{FF}), \quad (12)$$

i.e. at fixed p, f and s, V/v will be a linear function of V.

When $V/v = 1$,

$$(k_2/k_1 s)\,(1 + p/K_P + f/K_F + fp/K_P K_{FF}) = -(V/k_1 se)\,(1 + p/K_P + f/K_F + fp/K_P K_{FF}),$$

i.e. $$-V = k_2 e, \quad (13)$$

i.e. the extrapolated value of V obtained by continuing the straight line obtained by plotting V/v against V to the value $V/v = 1$ will be equal to $-k_2 e$.

Returning to Eqn. (5),

$$d/cp = 1/K_{FF},$$

i.e. $$d = cp/K_{FF} = (pf/K_F K_{FF})\, aK_m/s,$$

substituting the value for c, given by Eqn. (8). But, by Eqn. (9),

$$d = (pf/K_P K_{FF})\, aK_m/s.$$

Therefore $$K_F K_{FF} = K_P K_{FF}. \quad (14)$$

The Action of Thrombin on Fibrinogen

By L. LORAND and W. R. MIDDLEBROOK
Department of Biomolecular Structure, University of Leeds

(*Received* 31 *January* 1952)

A number of workers in the field of blood coagulation (Schmidt, 1895; Mellanby, 1908; Hammarsten, 1914; Nolf, 1938) have sought to explain the conversion of fibrinogen to fibrin in terms of a proteolytic change. Though this concept has been contradicted recently (Jaques, 1938; Astrup, 1950), the fact that certain proteases, e.g. papain (Eagle & Harris, 1937) and some snake venoms (Eagle, 1937) can bring about clotting served to uphold the idea. However useful the information gained by analogy from other enzymes, direct proof was necessary to show that thrombin acts in a similar manner. It was therefore desirable to undertake a systematic comparison of the free amino groups of both fibrinogen and fibrin. Sanger's (1945) method was used because of its high sensitivity and usefulness in the identification of the amino-acids involved.

A preliminary account of part of this work has already been published in conjunction with Dr K. Bailey and Mr F. R. Bettelheim (Bailey, Bettelheim, Lorand & Middlebrook, 1951), who worked independently.

EXPERIMENTAL

Materials

Fibrinogen. Since there is no real criterion of purity for fibrinogen, several methods of preparation were tried which were known to produce material of clottability exceeding 90%. In most cases fresh bovine plasma collected in 0·2% (w/v) sodium oxalate was used as the source of fibrinogen.

(I) Bovine plasma was precipitated with 0·25 saturated $(NH_4)_2SO_4$, the precipitate was centrifuged and redissolved in one-third of the original plasma volume of 0·9% (w/v) NaCl containing 0·2% (w/v) sodium oxalate, and the solution was then filtered. The precipitation with 0·25 saturated $(NH_4)_2SO_4$ was repeated three times, and the final precipitate was dissolved in 0·9% (w/v) NaCl and dialysed at 2° against a similar solution for 48 hr. It was then filtered through paper pulp which had been washed with 0·9% (w/v) NaCl.

(II) The first precipitation with $(NH_4)_2SO_4$ was the same as in the previous case; the separated precipitate was dissolved in one-third of the original plasma volume of 0·9% (w/v) NaCl and was filtered through paper pulp. The solution was then cooled to 0° and diluted to 10 vol. with cold distilled water. The fibrinogen was precipitated by the addition

of N-acetic acid to pH 5·5, centrifuged and redissolved in one-tenth of the original plasma volume of 0·9% (w/v) NaCl and neutralized by the addition of NH_4OH to pH 7·0–7·5 (Bagdy's (1948) method: personal communication).

(III) Bovine fraction I (Armour) (1 g.) was dissolved in 100 ml. 0·05M-phosphate buffer (KH_2PO_4:Na_2HPO_4 = 2:1). The solution was left in a refrigerator overnight and then filtered through paper pulp. One-third of saturated $(NH_4)_2SO_4$ was added to the filtrate and the precipitate was centrifuged, redissolved in 40 ml. 0·3M-KCl solution made slightly alkaline (pH 7·5) by the addition of $NaHCO_3$, then dialysed in the cold for 60 hr. against 0·2M-KCl. The freshly dialysed solution was finally filtered through a fine paper (Laki, 1951).

(IV) A sample of highly pure fibrinogen was made available by the courtesy of Prof. Seegers (no. 501020).

Thrombin. Hoffmann-La Roche and Seegers' purest thrombin preparations, known to be non-fibrinolytic, were used. Both were obtained as gifts.

Fibrin. This was isolated from any unchanged fibrinogen and from occlusions in the clot network by means of its solubility in urea (Lorand, 1948, 1950). The samples of fibrinogen were clotted with minute amounts of thrombin, the clot was dissolved in 30% (w/v) urea, and the solution was then poured into a large volume of 0·9% (w/v) NaCl solution, whereupon the fibrin separated out leaving any occluded material in solution. It is necessary to reduce the concentration of urea as much as possible, and certainly below 0·5% (w/v). The precipitated fibrin was washed thoroughly first with 0·9% (w/v) NaCl solution, then with water. (This method of recovering fibrin forms the basis of a newly devised clotting test; Lorand, 1951*a*.)

The clot system, consisting of fibrin and clot liquor, was also examined. Light's 1-fluoro-2:4-dinitrobenzene (FDNB) was used, and all the other reagents were of analytical purity.

Methods

Treatment with FDNB. The proteins were treated with FDNB under the conditions recommended by Sanger (1945), and the reaction was carried out at room temperature for 16–24 hr. This ensured complete penetration and reaction of FDNB even in the case of fibrin and the whole clot system. The treated proteins were washed with water, ethanol and ether and were dried at 110°.

Hydrolysis and separation of dinitrophenylamino-acids (DNP-acids). About 200 mg. of the treated proteins were hydrolysed under reflux in 20 ml. 5·7N-HCl. When DNP-glycine was being investigated, a compromise had to be effected between liberation and hydrolytic breakdown of this labile compound; thus the times of hydrolysis were set at 6, 8 and 12 hr. In the case of more stable DNP-acids, 24 and 48 hr. were chosen for times of hydrolysis.

The hydrolysate was shaken with peroxide-free ether and the DNP-acids which were ether-soluble were examined first by Sanger's method (1945). Extensive use was made of the buffered columns recommended by Middlebrook (1949, 1950, 1951). Columns were usually made from 2 g. of silica-gel by using 0·5 ml. of the stationary phase per 1 g. of silica. The DNP-acids were identified by measuring their R values on various columns in conjunction with various solvents, and comparing with the R values given by authentic samples of standard DNP-acids on the same columns.

The ether-soluble portion was estimated by evaporating the solution to dryness in a flask, and the residue was dissolved in a small amount of $CHCl_3$ and run on a pH 6·0 column with $CHCl_3$. The fast-moving decomposition products, including 2:4-dinitroaniline, were removed, but all the DNP-acids remained at the top of the column. The flask was rinsed with a small amount of ether, which was transferred to the column and the mobile solvent was changed to ether. Several bands were formed with R values that allowed the separation of a number of DNP-acids, e.g. bis-DNP-tyrosine was collected in the fast band, DNP-glycine moved with R = 0·5 and DNP-glutamic acid with R = 0·08. When DNP-threonine, -serine and -aspartic acid were present, they gave R values of 0·3, 0·2 and 0·02, respectively. In general, further identification and separation of the collected bands was carried out on other columns suitable for their characterization. The bis-DNP-tyrosine was passed through an unbuffered column with $CHCl_3$ (R = 0·4), DNP-glycine was run on an unbuffered column with $CHCl_3$ (R = 0·2), and DNP-glutamic acid with 1% (v/v) butanol-$CHCl_3$ (R = 0·1) on an unbuffered column.

The material remaining in the acid phase was estimated by drying down a portion of the ether-extracted hydrolysate, then taking it up in 30% (v/v) butanol-$CHCl_3$ and passing it through a silica-gel column with water as the stationary phase. In the experiments with fibrinogen and fibrin, only one band could be observed on this column corresponding to ϵ-*N*-DNP-lysine (R = 0·14).

Estimation of DNP-acids. The separated DNP-acids were made up to a volume of 10 ml. in the appropriate solvent and estimated by using a Beckman ultraviolet spectrophotometer. ϵ-*N*-DNP-lysine was measured in a solution of N-HCl, and the ether-soluble DNP-acids in a solution of 1% (w/v) $NaHCO_3$, and the optical densities were compared with calibrated values of standard DNP-acids. Readings of the ether-soluble DNP-acids were taken at 350 and 360 mμ., where they show maximal absorption; ϵ-*N*-DNP-lysine, however, was read at 390 mμ. as recommended by Sanger (1949). At concentrations less than 60 μM absorptions of the DNP-acids investigated obey Beer's law and measurements were taken within this limit of concentration.

Rate of decomposition of DNP-acids. By estimating the free DNP-acids after hydrolysing, for different lengths of time, portions of the same batch of treated protein, it is possible to measure the decomposition rates of the DNP-acids (Middlebrook, 1950, 1951). The general procedure for separating the DNP-acids was exactly the same as described above. Table 1 shows the hydrolytic breakdown of the DNP-acids derived from fibrinogen and fibrin. It seems that DNP-acids which are relatively stable in one system may be more labile in another. While the rate of decomposition found for DNP-glycine is close to that given by Sanger for insulin (Sanger, 1945) and by Middlebrook for wool (Middlebrook, 1950, 1951), the DNP-glutamic acid proved to be considerably more stable in the present case than in those reported by Porter & Sanger (1948) and by Middlebrook (1950, 1951). In view of the small amount of DNP-aspartic acid obtained from the treated fibrinogen the destruction rate of this acid was not determined, but the correction factor of Porter & Sanger (1948) was used.

The yields of DNP-glycine, corrected for loss by hydrolysis, were found to be the same after 8 and 12 hr. hydrolysis of the treated fibrin, but lower after only 6 hr. hydrolysis. Accordingly, in accurate determinations of the labile DNP-glycine, 8 hr. was chosen for hydrolysis in order to compromise between reasonable breakdown and as complete hydrolysis as possible.

Amide N *determination.* It was necessary to know the protein content of the treated fibrinogen and fibrin, and this was computed indirectly by estimating the amide N of the treated protein and comparing it with the value obtained for the untreated protein (Sanger, 1945). A mild hydrolysis was used with 2N-HCl for 4 hr., and after neutralization the liberated NH_3 was estimated in the micro-Kjeldahl apparatus as recommended by Sanger (1945). The amide N was found to be 1·30% for fibrinogen and 1·31% for fibrin, in close agreement with the values given by Bailey (1944).

Table 1. *Hydrolytic breakdown of DNP-acids by boiling in* 5·7N-HCl

Amino-acid derivative	Time of hydrolysis (hr.)	Amount unchanged (%)
DNP-glutamic acid	24	80
	24	81
	24	85
DNP-glycine	4	56
	16	21
ε-*N*-DNP-lysine	24	93
	24	94
Bis-DNP-tyrosine	16	81
	24	67
	40	55
	40	59

RESULTS

Fibrinogen. The different preparations examined for *N*-terminal groups gave essentially the same results in that glutamic acid and tyrosine (the latter was first observed by Bailey, 1951) were the chief terminal residues in all of them. In some preparations variable traces of glycine end groups were found, but these may be ascribed either to impurities or, more probably, to traces of fibrin (see below). In most of the samples listed, other *N*-terminal groups as well, including those of aspartic acid, serine and threonine, were detected in relatively small and varying proportions. One may safely assume that the presence of the last-named is due to impurities. In one of the purest preparations of fibrinogen, apart from glutamic acid and tyrosine, only aspartic acid could be found as an *N*-terminal residue and in very minute proportion. Table 2 illustrates the *N*-terminal groups probably related to impurities in the various fibrinogen preparations examined.

Fibrin. The samples of purified fibrin contained glycine and tyrosine *N*-terminal groups and in all cases were free from traces of other end groups.

Table 2. *N-Terminal groups probably related to impurities*

Fibrinogen sample	
I	Traces of glycine, serine, threonine, aspartic acid
II	Traces of glycine, serine, threonine, aspartic acid
III	Traces of threonine, aspartic acid
IV	Very minute traces of aspartic acid

The clot system. To obtain strict comparisons and also to make equal allowance for possible impurities, experiments in parallel were undertaken with fibrinogen and with the whole fibrin-plus-supernatant system obtained from it. The clot was found always to contain a considerable number of *N*-terminal groups of glycine, whereas none or only very few were found in fibrinogen. Also the number of glutamic acid *N*-terminal groups in the clotted protein was greatly reduced as compared with that of the unclotted material..

The results of the threefold approach outlined above are summarized in Tables 3 and 4.

Table 3. *Estimated number of moles of α-DNP-acids per* 450000 *unit of protein, after correction for hydrolytic breakdown (time of hydrolysis in hours in brackets)*

(Values in this table were derived from the same batch of fibrinogen. Where more than one value is given, these were obtained from different hydrolysates.)

	α-DNP-aspartic acid	α-DNP-glutamic acid	α-DNP-glycine	Bis-DNP-tyrosine
Fibrinogen IV	0·15 (24)	0·9 (24) 0·9 (24)	Absent (8)	2·0 (8) 2·0 (48)
Clot	Absent (12)	Traces (12)	4·0 (8) 4·1 (12)	Present, not estimated (24)
Purified fibrin	Absent (24)	Absent (24)	4·9 (8)	2·0 (8) 2·1 (24) 1·9 (48)

Table 4. *Lysine content derived from the ε-N-DNP-lysine estimations after* 24 *hr. hydrolysis*

(Values in this table were derived from the same batch of fibrinogen, preparation IV. The two values given for fibrinogen were obtained from one hydrolysate.)

	Lysine/100 g. protein (g.)	Lysine N as % of total protein N	Moles lysine/ 450000 unit of protein
Fibrinogen	6·32	7·17	195
	6·36	7·22	196
Clot	6·27	7·12	193
Purified fibrin	6·68	7·58	206

DISCUSSION

It is concluded from these results that fibrinogen contains *N*-terminal groups of glutamic acid and tyrosine, while fibrin has *N*-terminal groups of glycine and tyrosine. The quantitative values indicate that in a unit weight of 450000, fibrinogen has one glutamic acid and two tyrosine *N*-terminal residues (thus such a unit possesses three open peptide chains). It is believed that the small amount of terminal aspartic acid is due to impurities in the fibrinogen samples, since only one aspartic acid *N*-terminal group would be present in 5–10 times the unit weight chosen. A value of 450000 is in close agreement with published values for the molecular weight of fibrinogen (Edsall, Foster & Scheinberg, 1947). The same unit of fibrin ends in two tyrosine and presumably four glycine *N*-terminal residues.

An important conclusion can be drawn from these results with regard to changes that occur during the fibrinogen-fibrin transformation, namely that α-amino groups of glycine are liberated, possibly by the fission of glycyl-peptide bonds within the molecule. Such a change is a strong argument in favour of the proteolytic nature of the clotting mechanism. Bailey & Bettelheim (Bailey *et al.* 1951) have shown, in addition, that the action of thrombin seems specific for fibrinogen, since incubation of proteins such as ovalbumin and myosin with thrombin does not result in the liberation of new *N*-terminal groups; they have also proved that denaturation of fibrinogen does not bring about the appearance of glycine end groups. It is necessary to point out that our results and conclusions refer only to bovine fibrinogen; the structure of fibrinogen and the enzymic changes which take place on clotting may be species specific. Also, though the action of papain and proteolytic snake venoms on fibrinogen is known to result in clot formation, it does not follow that the changes at the molecular level are identical with those brought about by thrombin.

It can be seen from the results that the glutamic acid *N*-terminal residues present in fibrinogen could not be found by the FDNB method in the resulting fibrin. It was reasonable to suppose that the 'disappearance' of glutamic acid end groups was due to a splitting-off of a portion of the fibrinogen molecule which was removed as an α-DNP-glutamyl-peptide during washing after the FDNB treatment. Indeed, this assumption prompted the investigations which led to the discovery of fibrino-peptide (Lorand, 1951*a*, *b*, 1952).

Within the limits of experimental error fibrinogen and fibrin contain the same number of free ϵ-*N* groups of lysine as revealed by the FDNB method. Our values are somewhat lower than those found by Bailey (1951), who arrived at interesting conclusions with regard to the possible role of unreactive lysine side chains in the aggregation of fibrin particles.

SUMMARY

1. Bovine fibrinogen and fibrin were treated by the dinitrophenyl-method and the *N*-terminal residues determined.

2. In the fibrinogen molecule two chains end in tyrosine and one in glutamic acid. The same unit weight of fibrin has two *N*-terminal residues of tyrosine and four of glycine.

3. It was shown that the action of thrombin on fibrinogen results in the fission of glycyl-peptide bonds within the molecule, with the formation of fibrin and, it is suggested, the simultaneous removal of *N*-terminal residues of glutamic acid as part of a peptide.

4. Fibrinogen and fibrin contain the same number of free ϵ-*N* groups of lysine.

We wish to express our thanks to Prof. W. T. Astbury, F.R.S., for his interest and encouragement.

REFERENCES

Astrup, T. (1950). *Advanc. Enzymol.* **10**, 1.
Bagdy, D. (1948). Personal communication.
Bailey, K. (1944). *Advanc. Protein Chem.* **1**, 289.
Bailey, K. (1951). *Biochem. J.* **49**, 23.
Bailey, K., Bettelheim, F. R., Lorand, L. & Middlebrook, W. R. (1951). *Nature, Lond.*, **167**, 233.
Eagle, H. (1937). *J. exp. Med.* **65**, 613.
Eagle, H. & Harris, T. N. (1937). *J. gen. Physiol.* **20**, 543.
Edsall, J. T., Foster, J. F. & Scheinberg, H. (1947). *J. Amer. chem. Soc.* **69**, 2731.
Hammarsten, O. (1914). *A Textbook of Physiological Chemistry*, 7th English ed. New York: Wiley.
Jaques, L. B. (1938). *Biochem. J.* **32**, 1181.
Laki, K. (1951). *Arch. Biochem. Biophys.* **32**, 317.
Lorand, L. (1948). *Hung. Acta physiol.* **1**, 192.
Lorand, L. (1950). *Nature, Lond.*, **166**, 694.
Lorand, L. (1951*a*). Thesis. University of Leeds.
Lorand, L. (1951*b*). *Nature, Lond.*, **167**, 992.
Lorand, L. (1952). *Biochem. J.* **52**, 200.
Mellanby, J. (1908). *J. Physiol.* **38**, 28.
Middlebrook, W. R. (1949). *Nature, Lond.*, **164**, 501.
Middlebrook, W. R. (1950). Thesis. University of Leeds.
Middlebrook, W. R. (1951). *Biochim. Biophys. Acta*, **7**, 547.
Nolf, P. (1938). *Medicine*, **17**, 381.
Porter, R. R. & Sanger, F. (1948). *Biochem. J.* **42**, 287.
Sanger, F. (1945). *Biochem. J.* **39**, 507.
Sanger, F. (1949). *Biochem. J.* **45**, 563.
Schmidt, A. (1895). *Weitere Beiträge zur Blutlehre.* Wiesbaden: Bergmann.

Fibrino-peptide

By L. LORAND
Department of Biomolecular Structure, University of Leeds

(*Received* 31 *January* 1952)

Investigations on the *N*-terminal groups of fibrinogen and fibrin, as described in previous papers (Bailey, Bettelheim, Lorand & Middlebrook, 1951; Lorand, 1951*a*; Lorand & Middlebrook, 1952), revealed that *N*-terminal residues of glycine are set free in the fibrinogen molecule as a result of the action of thrombin. Whatever the underlying mechanism of clotting, it was shown that the fundamental enzymic step in the transformation of fibrinogen to fibrin is closely linked up with the liberation of these end groups. This was the first direct evidence that thrombin acts as a protease. Assay of the *N*-terminal residues showed, furthermore, that the glutamic acid end groups of the fibrinogen molecule were absent in fibrin. Such a change implied that their 'disappearance' during clotting was due, if they were not recombined in some way, to a splitting-off of part of the fibrinogen molecule.

Current ideas on the enzymic action of thrombin are governed by the conclusions of Jaques (1938), who stated that all fibrinogen nitrogen was converted into fibrin nitrogen. These findings (Astrup, 1950) do not favour the concept that the fibrinogen-thrombin reaction produces another substance besides fibrin. It became clear, however (Lorand, 1951*a*, *b*), that Jaques's approach in studying the *N*-partition of the clotting system cannot be regarded as adequate because of the possibility of a second reaction product being occluded within the clot and so being estimated as fibrin. If such a substance is produced in the clotting system, it will not be detected unless looked for in a special way. A short account of the work presented here in detail has already been published (Lorand, 1951*b*).

MATERIALS

Fibrinogen. This was purified from commercial Bovine fraction I (Armour) by precipitation with 0·25 vol. saturated $(NH_4)_2SO_4$ (Laki, 1951*a*). The clottability of the final product exceeded 90%, and care was taken that only freshly dialysed solutions were used in the experiments.

Thrombin. This was obtained by the courtesy of Prof. Seegers and was used in aqueous, freshly dialysed solutions (2 mg./ml.).

Other reagents were of analytical purity, and glass-distilled water was used throughout the experiments.

EXPERIMENTAL AND RESULTS

The release of non-protein N from the fibrinogen-thrombin system

The fibrin gel could be converted into a flocculent precipitate by dissolving it in 1% (w/v) monochloroacetic acid and then precipitating the proteins with 7% (w/v) trichloroacetic acid. This allows the release of non-protein material in the supernatant which was assayed for nitrogen by the micro-Kjeldahl method. Monochloroacetic acid, like urea (Lorand, 1950*a*, 1951*a*), dissolves the clot rapidly to produce a clear solution. It neither interferes with the nitrogen estimation nor does it inhibit the deproteinizing action of trichloroacetic acid.

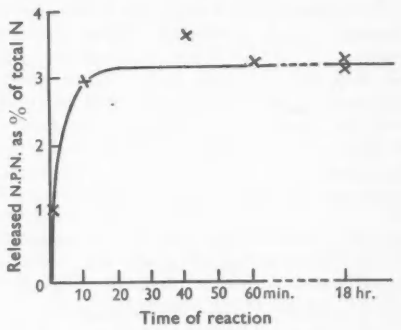


Fig. 1. Increase of N.P.N. (% of total N) with time of action of thrombin on fibrinogen.

In typical experiments the following procedure was adopted. To 8 ml. portions of fibrinogen (0·378 mg. N/ml.) dialysed against 0·2M-KCl, 2 ml. 0·05M phosphate buffer, pH 6·8, and 0·2 ml. thrombin were added. Clotting occurred within 30 sec., and the gels were dispersed at different times with 9·8 ml. of 2% (w/v) monochloroacetic acid. After dissolution of the clots in 2 min., 10 ml. of 20% (w/v) trichloroacetic acid were added. Half an hour after the precipitation of the proteins the mixtures were filtered and N was estimated in duplicate on suitable portions of the filtrates. Kjeldahl digestions were carried out for 8 hr. using the catalyst mixture recommended by Chibnall, Rees & Williams (1943), and the method of estimating N was that of Ma & Zuazaga (1942). A solution of fibrinogen,

to which mono- and tri-chloroacetic acid was added prior to thrombin, was used as control. The results are shown in Fig. 1.

The asymptotic level of the time-curve, maintained for more than 18 hr., indicates that the increase of non-protein nitrogen (N.P.N.) in the supernatant is not due to fibrinolysis, but is a characteristic feature of the enzymic action of thrombin on fibrinogen. The reaction does not stop with the onset of gelation, but goes on to completion when about 3% of the total N appears as N.P.N. This implies that a pure fibrinogen preparation cannot contain more than about 97% clottable N if fibrin could be recovered by an adequate method. (For an attempt along such lines see Lorand, 1951*a*.)

Peptide liberated by the action of thrombin on fibrinogen

Filtrates of deproteinized solutions of fibrinogen and fibrin were shaken with ether in order to remove the chloroacetic acids. Portions (1 ml.) of each of the ether-extracted solutions were dried *in vacuo* and hydrolysed with 0·2 ml. 5·7 N-HCl in sealed tubes for 24 hr. at 110°. After the acid had been evaporated, both hydrolysates were subjected side by side on the same paper strip to one-dimensional ascending chromatography. Phenol-NH_3 was used as mobile phase, and the chromatograms were developed with a freshly made solution of 0·1% (w/v) ninhydrin in ether, heated for 5 min. at 100°.

The chromatogram corresponding to the hydrolysate of the protein-free supernatant of fibrinogen (repeated on three different preparations) showed a complete absence of amino-acids; while that representing the protein-free supernatant of fibrin produced several spots of varying intensity.

In later experiments, in order to avoid the distortion of the chromatograms by the presence of salts, the original fibrinogen solution was dialysed against a solution of 0·05% (w/v) ammonium carbonate. Clotting could be conducted adequately in this medium, and the ammonium carbonate was volatilized during subsequent steps of the experiment. The results obtained by this modified process were substantially the same as described above, and the quality of the chromatograms was improved.

The R_F values of the amino-acids in the hydrolysate from the protein-free supernatant of fibrin were found, with phenol-ammonia as the mobile phase on a one-dimensional chromatogram, to be as follows:

R_F values	0·1	0·29	0·4	0·5	0·65	0·87	0·92
Relative intensity of colour	Strong	Strong	Weak	Strong	Weak	Weak	Weak

Chromatograms of unhydrolysed samples of supernatants did not show spots corresponding to free amino-acids.

Isolation of peptide from the fibrinogen-thrombin system

The fibrinogen was thoroughly dialysed against a solution of 0·05% (w/v) $(NH_4)_2CO_3$ at 0° until all other salts were replaced. Thrombin was mixed with the freshly dialysed fibrinogen solution at room temperature, so that the clotting time was 1–2 min. 2 hr. after gelation an equal volume of 2% (w/v) monochloroacetic acid was added, whereupon the clot dissolved rapidly. When a clear solution was obtained, the proteins were precipitated by addition of half a volume of 20% (w/v) trichloroacetic acid. The precipitate was allowed to settle and the mixture was then filtered. The filtrate was concentrated by vacuum distillation at about 13° into a smaller volume. This solution was thoroughly extracted with peroxide-free ether in order to remove the chloroacetic acids. The ether-extracted solution was then evaporated *in vacuo* and the separated material washed with ether.

The substance obtained was readily soluble in water; the solution gave no precipitate on adding trichloroacetic acid, showing that no protein was present. A paper chromatogram of the substance demonstrated the absence of free amino-acids. The acid hydrolysate of the material, however, gave rise to a number of amino-acid spots on the chromatogram, similar to those described in the previous section.

The peptide can be precipitated from its solution in water by 75% (v/v) acetone, and by this method an apparently pure sample can be obtained. Investigations for establishing the composition and molecular structure of the peptide are being carried out and will be reported on later. Preliminary results (Lorand & Middlebrook, 1951) indicate that the free amino end of the peptide chain consists of a glutamic acid residue, in good agreement with the previously reported finding that the *N*-terminal glutamic acid groups of the fibrinogen molecule are absent in fibrin (Lorand, 1951*a*; Lorand & Middlebrook, 1952). From a personal communication it is learnt that Dr K. Bailey and Mr F. R. Bettelheim (1951), in parallel experiments at Cambridge, have also isolated a homogeneous peptide from the clot liquor. They have prepared this peptide both in the free form and also as a water-soluble dinitrophenyl compound; its terminal amino groups account for the difference found in the content of glutamic acid *N*-terminal groups between fibrinogen and fibrin.

According to private communications, Laki (1951*b*) noticed recently the appearance of a substance in the protein-free supernatant of the iodinated fibrinogen and thrombin system. The supernatant was found to be biologically active in stimulating the frog heart (Laki, 1951*c*), and it is known now to give the biuret reaction. It is believed,

therefore, that the substance when isolated may turn out to be identical with the peptide reported previously by the present author (Lorand, 1951*a*, *b*).

DISCUSSION

Previous authors (Bailey *et al.* 1951; Lorand, 1951*a*; Lorand & Middlebrook, 1952) had reported that peptide bonds involving the amino group of glycine are split by thrombin within the fibrinogen molecule when it is converted to fibrin, and it had also been shown that the glutamic acid *N*-terminal residues of fibrinogen cannot be found in fibrin. The present paper reports further that the fibrinogen-fibrin transformation is accompanied by a simultaneous liberation of N.P.N., and evidence is presented to show that a peptide is set free at the same time. As suggested in the preliminary note (Lorand, 1951*b*), this peptide should be called 'fibrino-peptide', for it is believed that it is produced by splitting off a portion of the fibrinogen molecule during the formation of fibrin. The possibility remains that the free glutamic acid *N*-terminal residues always present in fibrinogen preparations are due to a tenaciously adsorbed peptide which is released only if the underlying molecule is converted to fibrin; but in view of the uniform results obtained with various preparations, this cannot be a likely explanation. The facts that (*a*) the peptide is not released from the fibrinogen preparation without the action of thrombin, (*b*) thrombin has been shown to be a specific protease, (*c*) the *N*-terminal residues of glutamic acid of the original fibrinogen are found as end groups of the released peptide, and (*d*) the liberation of N.P.N. in the fibrinogen-thrombin system proceeds parallel with clotting without the occurrence of fibrinolysis, are all strong arguments in favour of 'fibrino-peptide' being derived by fission of the fibrinogen molecule during its transformation into fibrin. Further evidence to establish the relationship between the release of fibrino-peptide and the clotting mechanism is being sought by studying the molecular structure of the peptide.

Since it appears that fibrin is not the sole reaction product of the enzymic action of thrombin on fibrinogen, the process can accordingly be represented as follows:

$$\text{Fibrinogen} \xrightarrow{\text{Thrombin}} \text{Fibrin} + \text{fibrino-peptide}.$$

The reactions occurring when pepsinogen is activated by pepsin (Herriott, 1941), or when ovalbumin is converted to plakalbumin by a bacterial enzyme (Linderstrøm-Lang & Ottesen, 1949), must be very similar to the fibrinogen-fibrin transformation. In all three cases a new protein species is created merely by splitting off a peptide or a few amino-acid fragments from the main body of the original substance.

$$\text{Protein A} \xrightarrow{\text{Enzyme}} \text{Protein B} + \text{low-molecular substances (peptides)}.$$

The resulting product (protein B) displays quite different behaviour from that of the primary protein, though differing so little from it in molecular constitution. The concept of such a relatively minor change of the fibrinogen molecule during clotting would explain the findings that there is no appreciable difference between the viscosity of fibrinogen and fibrin in urea (Lorand, 1948; Lorand, 1950*a*, *b*; Mihalyi, 1950*a*), and that both proteins have identical sedimentation constants in urea solution (Shulman, Ehrlich & Ferry, 1951). It should also be noticed that differences in the electrophoretic mobilities and isoelectric points of fibrinogen and fibrin (Mihalyi, 1950*b*) can most readily be interpreted in terms of a proteolytic change involving fission of a peptide from the fibrinogen.

The proposed mechanism of the enzymic step in the clotting of fibrinogen (Lorand, 1951*a*, *b*), as outlined above, is supported by the recent observations of Laskowski, Rakowitz & Scheraga (1952). These authors showed that the reaction

$$\text{Fibrinogen} \xrightarrow{\text{Thrombin}} \text{Fibrin} + \text{fibrino-peptide}$$

is a reversible one, and they demonstrated that the equilibrium could be shifted to the left by adding fibrino-peptide to the system.

'Non-specific' clotting agents of the proteolytic type (papain, and certain snake venoms) are known to produce clots which, like fibrin, are soluble in urea (Janszky, 1949; Lorand, 1951*a*); but it is not known yet if these enzymes cause the same molecular alteration of fibrinogen as thrombin does, namely the liberation of glycine *N*-terminal groups with the splitting off of fibrino-peptide. The type of the reaction may be similar, but different enzymes may show individual variations in their actions at the molecular level. It is conceivable that in the course of the continuous degradation of fibrinogen by these agents a transitory stage is reached when the composition of the molecule happens to be similar to that of true fibrin, so that the particles then associate to a clot which, as the non-specific enzyme activity proceeds, is eventually digested. The action of thrombin, however, seems to be limited by its high specificity simply to producing fibrin.

SUMMARY

1. It is shown that the clotting activity of thrombin, known to be a specific protease, is accompanied by the liberation of non-protein nitrogen.

2. A peptide appears in the fibrinogen-thrombin system.

3. It is suggested that this hitherto unknown substance should be called 'fibrino-peptide', since it is believed to be derived by splitting off part of the fibrinogen molecule.

4. A method for isolating apparently pure fibrino-peptide is given.

5. The release of fibrino-peptide is correlated with previous results, and the clotting of fibrinogen is discussed in the light of the new finding.

I wish to thank Prof. W. T. Astbury, F.R.S., for the benefit of discussions, and Dr W. R. Middlebrook for his valuable help in the chromatographic experiments.

REFERENCES

Astrup, T. (1950). *Advanc. Enzymol.* **10**, 1.
Bailey, K., Bettelheim, F. R., Lorand, L. & Middlebrook, W. R. (1951). *Nature, Lond.*, **167**, 233.
Bailey, K. & Bettelheim, F. R. (1951). (Personal communication.)
Chibnall, A. C., Rees, M. W. & Williams, E. F. (1943). *Biochem. J.* **37**, 354.
Herriott, R. M. (1941). *J. gen. Physiol.* **24**, 325.
Janszky, B. (1949). *Science*, **110**, 307.
Jaques, L. B. (1938). *Biochem. J.* **32**, 1181.
Laki, K. (1951*a*). *Arch. Biochem. Biophys.* **32**, 317.
Laki, K. (1951*b*). (Personal communication.)
Laki, K. (1951*c*). *Science*, **114**, 435.
Laskowski, M. jun., Rakowitz, D. H. & Scheraga, H. A. (1952). *J. Amer. chem. Soc.* **74**, 280.
Linderstrøm-Lang, K. & Ottesen, M. (1949). *C.R. Lab. Carlsberg, Ser. chim.*, **26**, 404.
Lorand, L. (1948). *Hungarica Acta physiol.* **1**, 192.
Lorand, L. (1950*a*). *Nature, Lond.*, **166**, 694.
Lorand, L. (1950*b*). *Proc. int. Soc. Haematology*, p. 407.
Lorand, L. (1951*a*). Thesis. University of Leeds.
Lorand, L. (1951*b*). *Nature, Lond.*, **167**, 992.
Lorand, L. & Middlebrook, W. R. (1951). Unpublished results.
Lorand, L. & Middlebrook, W. R. (1952). *Biochem. J.* **52**, 196.
Ma, T. S. & Zuazaga, G. (1942). *Industr. Engng Chem.* (Anal. ed.), **14**, 280.
Mihalyi, E. (1950*a*). *Acta chem. scand.* **4**, 317.
Mihalyi, E. (1950*b*). *Acta chem. scand.* **4**, 344.
Shulman, S., Ehrlich, P. & Ferry, J. D. (1951). *J. Amer. chem. Soc.* **73**, 1388.

The Branched-Chain Fatty Acids of Mutton Fat

1. THE ISOLATION OF (+)-14-METHYLHEXADECANOIC ACID

By R. P. HANSEN, F. B. SHORLAND and N. J. COOKE
Fats Research Laboratory, Department of Scientific and Industrial Research, Wellington, New Zealand

(*Received* 24 *January* 1952)

Recent investigations in this laboratory have revealed the presence of branched-chain fatty acids as minor constituents of butterfat (Hansen & Shorland, 1950, 1951, 1952; Hansen, Shorland & Cooke, 1951*a*) and of beef fat (Hansen, Shorland & Cooke, 1952). The branched-chain acids isolated from butterfat comprised two C_{17} and one C_{18} saturated mono-methyl acids, and a saturated multi-methyl acid. From beef fat was derived a C_{17} methyl-substituted acid corresponding with one of the C_{17} acids isolated from butterfat.

Prior to the above findings, branched-chain acids had been located in the lipids of a number of bacilli (Anderson, 1927, 1941; Anderson & Chargaff, 1929; Velick, 1944*a*, *b*; Hofmann & Lucas, 1950) and in wool grease (Weitkamp, 1945), while the short-chain acid *iso*valeric was found by Chevreul in 1817 (cf. Hilditch, 1947) in the head and jaw oil of the dolphin.

The purpose of this paper is to describe in detail the isolation of (+)-14-methylhexadecanoic acid from the external tissue fat of ewes. The occurrence of this acid in ewe fat was briefly reported last year (Hansen, Shorland & Cooke, 1951*b*). Although (+)-14-methylhexadecanoic acid has been shown by Weitkamp (1945) to be present in wool grease, where it occurs as a sterol ester, and although Velick & English (1945) synthesized this acid and confirmed its structure, it has not hitherto been reported as a constituent of natural fats.

Since this manuscript has been prepared Dr G. Weitzel and Dr K. Thomas have, in a private com-

munication, referred to their discovery of optically active branched-chain higher fatty acids in the coccygeal glands of ducks and geese.

EXPERIMENTAL

The fat used in this investigation (saponification equiv. 286·9, iodine value 46·6, unsaponifiable matter 0·56% and free fatty acids 0·4%) was obtained by mincing and then steam-rendering the external tissue fats of old overweight ewes. In accordance with the procedure earlier outlined (Hansen & Shorland, 1951), methyl esters (7·69 kg.) were prepared, and after hydrogenation were crystallized repeatedly from acetone at $-30°$ to yield 332·6 g. of 'liquid' esters which were distilled in a 22-plate Vigreux column at about 0·2 mm. (cf. Table 1). Fractions HL10, HL11 and HL12 were bulked together, denoted H1, and fractionated *in vacuo* in a 40-plate column of a modified Lecky & Ewell (1940) type (cf. Table 2). Details of this column will be published elsewhere.

Table 1. *Fractionation of 'liquid' methyl esters from hydrogenated mutton fat*

(Iodine value 7·0.)

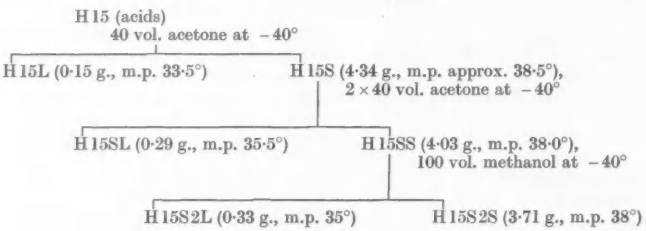
Fraction	Wt. (g.)	Saponification equiv.	Iodine value (Wijs)	
HL1	17·00	234·9	1·7	Liquid
HL2	22·64	240·5	0·7	Semi-solid
HL3	21·64	241·8	0·1	Solid
HL4	25·23	242·5	0·9	Solid
HL5	22·27	246·9	0·5	Liquid
HL6	21·48	256·0	1·1	Liquid
HL7	23·76	261·2	2·1	Liquid
HL8	21·74	265·1	2·4	Liquid
HL9	19·35	273·1	1·4	Liquid
HL10	22·65	279·6	5·8	Liquid
HL11	21·99	284·9	7·3	Liquid
HL12	25·87	287·8	3·3	Liquid
HL13	16·94	297·9	9·5	Solid
HLR	47·50	319·6	28·9	Viscous liquid
Total	330·06			

Methyl esters H1L2, H1L3, H1L4 from the above fractionation were combined, denoted H4, and crystallized as shown in Table 3.

Fraction H10S2L was further purified by dissolving the fraction in 300 ml. of light petroleum b.p. 50–60°, and subjecting it to chromatographic separation using 320 g. of activated alumina in a column of 3·5 cm. diam. (cf. Table 4).

Fractions H10S2Lc to H10S2Lg were combined (5·03 g.) and denoted H15, while fraction H10S2Lb (7·62 g.) was submitted to further chromatographic separation. The characteristics of fraction H15 were as follows:

Saponification equivalent 284·7, iodine value 1·4, m.p. 2·5°; m.p. (acid) 37·5°. The acids were crystallized as follows:


H15 (acids)
40 vol. acetone at −40°
H15L (0·15 g., m.p. 33·5°)
H15S (4·34 g., m.p. approx. 38·5°),
2 × 40 vol. acetone at −40°
H15SL (0·29 g., m.p. 35·5°)
H15SS (4·03 g., m.p. 38·0°),
100 vol. methanol at −40°
H15S2L (0·33 g., m.p. 35°)
H15S2S (3·71 g., m.p. 38°)

X-ray diffraction measurements reported in this work were made using a Philips Geiger X-ray spectrometer. Samples were melted on a glass slide and quickly cooled.

All melting points were determined in closed capillaries and are uncorrected.

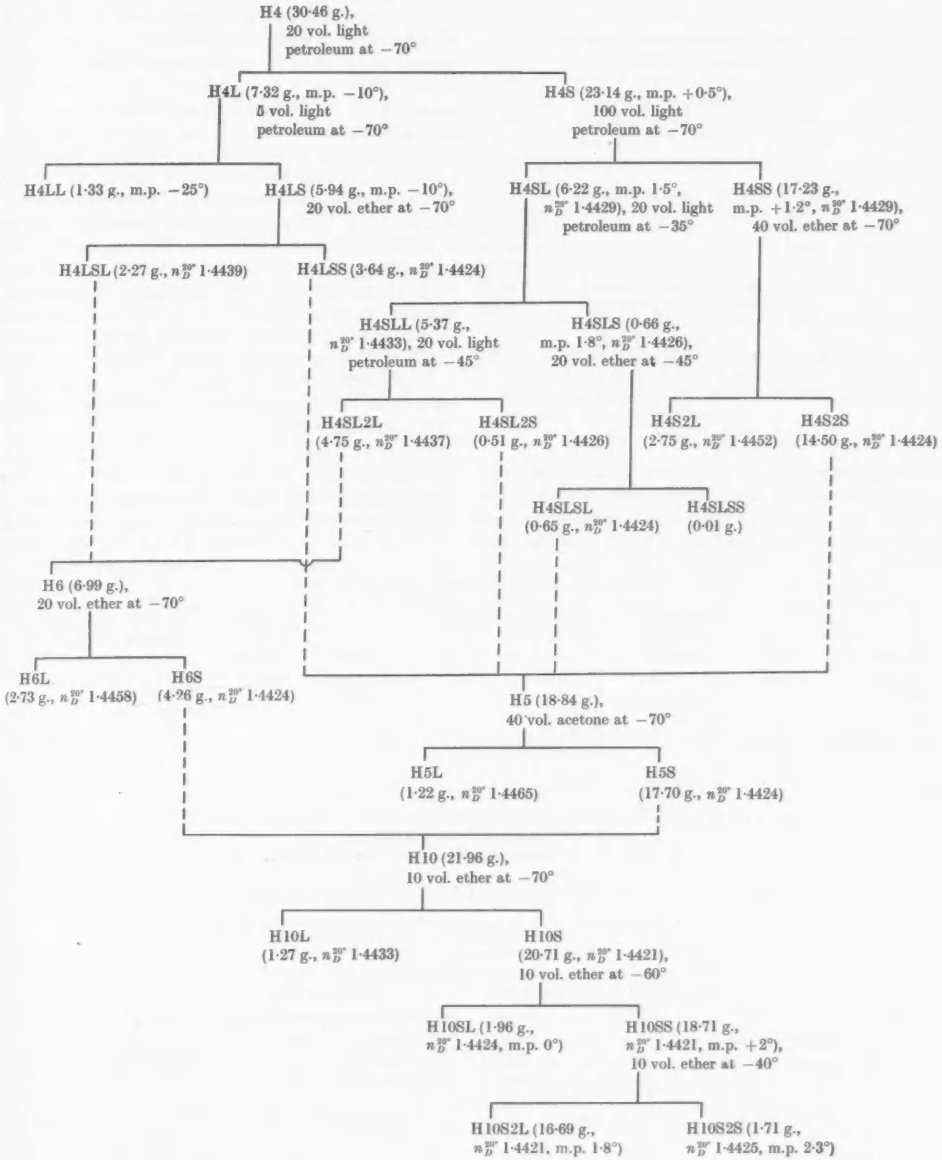
Table 2. *Refractionation of fractions HL10, HL11 and HL12 combined and denoted H1*

Fraction	Wt. (g.)	Saponification equiv.	Iodine value (Wijs)	
H1L1	12·39	276·6	1·2	Liquid
H1L2	11·34	283·5	0·8	Liquid
H1L3	11·57	284·1	1·4	Liquid
H1L4	6·26	284·7	0·6	Liquid
H1L5	11·16	282·6	1·5	Liquid
H1LR	12·22	301·1	11·7	Liquid
Total	64·94			

DISCUSSION

The saponification equivalent and the combustion analysis of fraction H15S2S agree closely with the theoretical values for a saturated fatty acid with empirical formula $C_{17}H_{34}O_2$. The *C*-methyl value, however, which is equivalent to 1·55 molecules of acetic acid, affords evidence of the presence of one side-chain methyl group (cf. Ginger, 1944). With an X-ray long spacing of 30·0 A. and a melting point of 38° this acid is further established as being distinct from normal heptadecanoic acid, m.p. 59·2–60·5 (Cason & Winans, 1950), X-ray long spacing 40·45 A. (Francis & Piper, 1939). From the melting point evidence adduced by Cason & Winans (1950) in their investigations on the methyl-substituted isomers of octadecanoic acid, the relatively high melting point of H15S2S (38°) suggests that the

Table 3. *Low-temperature crystallization of concentrate (H 4) of methyl esters of C_{17} acids*



side chain will not be located at the centre of the molecule, for in this position minimum melting points are shown to occur.

Table 4. *Chromatographic separation of fraction H 10S 2L*

Fraction	Wt. (g.)	Eluant	$n_D^{20°}$
H10S2La	0·03		Solid
b	7·62		1·4421
c	2·38		1·4417
d	1·12	300 ml. light petroleum	1·4419
e	0·73	(each fraction)	1·4417
f	0·52		1·4418
g	0·40		1·4418
h	0·28		1·4423
i	0·40	600 ml. light petroleum	1·4423
j	0·33	900 ml. light petroleum	1·4424
k	0·03	300 ml. light petroleum containing 5% ether	—
l	0·12	300 ml. light petroleum containing 10% ether	1·4431
m	0·01	300 ml. containing 50% light petroleum and 50% ether	—

Comparison of the properties of this optically active branched-chain C_{17} saturated acid fraction with those of the C_{17} dextrorotatory *ante-iso* acid isolated from wool grease by Weitkamp (1945) and subsequently synthesized by Velick & English (1945) identifies H 15S 2S as (+)-14-methylhexadecanoic acid (cf. Table 6).

Although Velick (1947) reported a value of 33·4 A. for the X-ray long spacing of (+)-14-methylhexadecanoic acid from wool grease, Nunn (1951), when analysing a sample of the same acid, also supplied by Weitkamp, observed an X-ray long spacing of 29·9 A. This latter figure corresponds closely with that reported in this paper. Nunn was unable to confirm Velick's (1947) value, but stated: 'It is possible that the method of preparation of the samples (for X-ray examination) used by us gave crystals in a metastable state.' The accuracy of the measurement recorded here (30·0 A) was established by determining the X-ray long spacing of a standard sample of palmitic acid which gave a value of 35·6 A., this figure being identical with the accepted value of Francis & Piper (1939).

The main fraction reported in this paper (H 15S 2S) comprises 3·71 g., but this quantity represents only a portion of the total content of this acid. Other fractions derived from H 10S 2L by chromatographic separation (cf. Table 4) also consisted mainly of this acid. For example, chromatography and fractional crystallization of H 10S 2Lb (7·62 g.) yielded a further 3·45 g. of acids, m.p.

Table 5. *Characteristics of fraction H 15S 2S*

Fraction	M.p.	Saponification equiv.	Combustion analysis*	Iodine value (Wijs)	C-Me*	X-ray long spacing	Specific rotation	Refractive index
H15S2S acid	38·0°	270·5 (Calc. for $C_{17}H_{34}O_2$: 270·4)	C, 75·7; H, 12·7% (Calc. for $C_{17}H_{34}O_2$: C, 75·5; H, 12·7%)	0·9	8·6% (equiv. to 1·55 mol. acetic acid)	30·0 A.	$[\alpha]_D^{23·2°}$ +3·83° ($CHCl_3$)	—
H15S2S methyl ester	2·5°	283·1 (Calc. for $C_{18}H_{36}O_2$: 284·5)	—	—	—	—	—	$n_D^{20°}$ 1·4424

* Analysis by Weiler and Strauss, Oxford, England.

Table 6. *Comparison of properties of fraction H 15S 2S with those of natural and synthetic forms of 14-methylhexadecanoic acid*

Sample	M.p.	X-ray long spacing (A.)	Optical rotation	
H15S2S	38·0°*	30·0	$[\alpha]_D^{23·2°}$ (in chloroform) +3·83°	This work
(+)-14-Methylhexadecanoic acid	—	33·4 *c*, 29·9 *d*	$[\alpha]_D^{26°}$ (in acetone) +5·0° *a*	Isolated by Weitkamp (1945) from wool grease
	36·8 *a*, 37·3–37·6 *b*	—	$[\alpha]_D^{26°}$ (in acetone) +5·23° *b*	—
(+)-14-Methylhexadecanoic acid	36–36·8° *b*	—	$[\alpha]_D^{20°}$ (in acetone) +5·16°	Synthesized by Velick & English (1945)
(±)-14-Methylhexadecanoic acid	39·5–40·1° *d*	29·7 *d*	—	Synthesized by Nunn (1951)

* Melting point uncorrected.

a, determined by Weitkamp (1945); *b*, determined by Velick & English (1945); *c*, determined by Velick (1947); *d*, determined by Nunn (1951).

37·5°. In the sample of external tissue fat analysed, (+)-14-methylhexadecanoic acid constitutes not less than 0·2% of the total fatty acids. Examination of other fractions from mutton fat is in progress, and the evidence already accumulated suggests that appreciable quantities of branched-chain acids are present.

The work now reported establishes that certain branched-chain acids isolated from wool grease (Weitkamp, 1945) and presumably secreted by the sebaceous glands of the sheep are also present in the external tissue fat, and possibly in the other fatty tissues of this animal.

SUMMARY

(+)-14-Methylhexadecanoic acid has been shown to occur as a minor constituent of mutton fat.

We wish to acknowledge the assistance given in this work by Mr M. Fieldes, Soil Bureau, Department of Scientific and Industrial Research, Wellington, who kindly carried out all X-ray measurements.

To Mr M. S. Carrie, Chemist to The Hawkes Bay Farmers Freezing Company Ltd., and to the management of that Company, we are indebted for arranging supplies of suitable material for this investigation.

REFERENCES

Anderson, R. J. (1927). *J. biol. Chem.* **74**, 525.
Anderson, R. J. (1941). *Chem. Rev.* **29**, 225.
Anderson, R. J. & Chargaff, E. (1929). *J. biol. Chem.* **85**, 77.
Cason, J. & Winans, W. R. (1950). *J. org. Chem.* **15**, 139.
Francis, F. & Piper, S. H. (1939). *J. Amer. chem. Soc.* **61**, 577.
Ginger, L. G. (1944). *J. biol. Chem.* **156**, 453.
Hansen, R. P. & Shorland, F. B. (1950). *J. N.Z. Inst. Chem.* **14**, 142.
Hansen, R. P. & Shorland, F. B. (1951). *Biochem. J.* **50**, 207.
Hansen, R. P. & Shorland, F. B. (1952). *Biochem. J.* **50**, 358.
Hansen, R. P., Shorland, F. B. & Cooke, N. J. (1951*a*). *Chem. & Ind.* p. 839.
Hansen, R. P., Shorland, F. B. & Cooke, N. J. (1951*b*). *J. N.Z. Inst. Chem.* (in the Press).
Hansen, R. P., Shorland, F. B. & Cooke, N. J. (1952). *Biochem. J.* **50**, 581.
Hilditch, T. P. (1947). *The Chemical Constitution of Natural Fats*, 2nd ed. revised, p. 392. London: Chapman and Hall.
Hofmann, K. & Lucas, R. A. (1950). *J. Amer. chem. Soc.* **72**, 4328.
Lecky, H. S. & Ewell, R. H. (1940). *Industr. Engng Chem.* (Anal. ed.), **12**, 544.
Nunn, J. R. (1951). *J. chem. Soc.* p. 1740.
Velick, S. F. (1944*a*). *J. biol. Chem.* **152**, 533.
Velick, S. F. (1944*b*). *J. biol. Chem.* **156**, 101.
Velick, S. F. (1947). *J. Amer. chem. Soc.* **69**, 2317.
Velick, S. F. & English, J. (1945). *J. biol. Chem.* **160**, 473.
Weitkamp, A. W. (1945). *J. Amer. chem. Soc.* **67**, 447.
Weitzel, G. & Thomas, K. Private communication, citing *Hoppe-Seyl. Z.* (1951), **288**, 251.

Seasonal Variations in Fatty Acid Composition of New Zealand Butterfat

By R. P. HANSEN and F. B. SHORLAND
Fats Research Laboratory, Department of Scientific and Industrial Research, Wellington, New Zealand

(*Received* 6 *February* 1952)

New Zealand butterfat has been shown by Cox & McDowall (1948) to exhibit seasonal variations in softening points, iodine values, saponification values and Reichert values. The characteristic trend of variation of each of these properties was observed to be remarkably uniform in the four different years reviewed, and in butterfats from different localities. Interpretation of the precise nature of these changes, however, requires a knowledge of the fatty acid composition and the glyceride structure of the different butterfats.

Many workers have determined the fatty acid composition of butterfat to elucidate its nature (Hilditch & Jones, 1929; Jack, Henderson & Hinshaw, 1946), to study the effect of diet on the milk fat (Hilditch & Sleightholme, 1930; Hilditch & Thompson, 1936; Hilditch & Jasperson, 1943) or to find the factors responsible for seasonal changes (Hilditch & Jasperson, 1941). A great deal of information has thus been accumulated, but so far as we are aware no systematic investigations have been made similar to the work now reported. It comprises both triplicate analyses of butterfat samples taken from the same locality at regular intervals over a whole year, and statistical evaluation of the results.

The purpose of this investigation was to measure statistically at regular intervals the fluctuations in the fatty acid composition of New Zealand butterfat and to attempt correlation of these changes in

chemical composition with the findings of Cox & McDowall (1948). Six butterfat samples, representing butter churned at two-monthly intervals, were analysed in triplicate to provide data for the comparison of fatty acid composition at regular intervals over the period of 1 year. For the comparison of fatty acid composition in corresponding months of different years, triplicate analyses of a further two samples are submitted. Still another analysis serves to indicate the composition of the fat in the relatively dry month of March. Of the ten butterfat samples described in this paper, nine were supplied by the Rangitaiki Plains Dairy Co. Ltd., Whakatane, and were representative of the bulk churning of cream from numerous outdoor pasture-fed herds in the district. (Jerseys constitute no less than 85% of New Zealand's dairy-cow population, and have resulted from crossing Jersey sires with Shorthorn, Friesian and Ayrshire cows (New Zealand Department of Agriculture, 1948).) The tenth sample (B/1) was supplied by the Dairy Research Institute and represented an April 1945 churning from a mixed herd of outdoor pasture-fed cattle at Massey Agricultural College, Palmerston North.

As reported in a paper on the accuracy of the ester fractionation analyses of butterfat (Hansen, 1951), the procedure adopted in these analyses does not presume to provide confirmation of the absolute accuracy of results. A measure of the degree of reproducibility of results, however, has been established by analysing all samples in triplicate and determining statistically the standard deviation for each constituent acid within each sample and over all samples. Statistical assessment has also been made of the significance of differences between butterfats churned in different months of the same year and between those churned at corresponding months of different years.

EXPERIMENTAL

All analyses recorded in this paper have been carried out by the ester-fractionation method, based on the conventional procedure of Hilditch (1947) and Hilditch & Jones (1929). Minor modifications adopted in this work have been reported by Hansen (1951). The method followed was as outlined below.

The fatty acids from 750 g. butterfat were steam-distilled and the steam-non-volatile acids separated into 'liquids' and 'solids' by crystallization three times from 15 vol. acetone at $-30°$. The steam-volatile acids were fractionated in a $60 \times 1{\cdot}7$ cm. electrically heated and packed column of the type described by Longenecker (1937), while the 'liquids' and 'solids' respectively were converted to methyl esters and distilled *in vacuo* in a $90 \times 3{\cdot}5$ cm. packed fractionating column, electrically heated in three sections, as described by Jack & Henderson (1945). The efficiency of this latter column was measured at equilibrium using a mixture of CCl_4 and benzene, and was estimated using the data of Coulson & Herington (1948) to possess seventeen theoretical plates.

Calculations involved in the resolution of distilled fractions into components have been based on saponification equivalents and iodine values, and in general have followed the principles adopted by Hilditch (1947). Nevertheless, to clarify interpretation of the results certain assumptions used in this work are specified below.

It has been customary (cf. Hilditch, 1947) to calculate the proportions of oleic and octadecadienoic acids from the iodine value of the C_{18} unsaturated acid fractions on the assumption that only these two acids are present, but recently it has been shown (Shorland, 1950) that in addition to diene acids there is present in New Zealand butterfat linolenic acid in somewhat similar proportions. We have, therefore, made no attempt to resolve the C_{18} unsaturated acids into their components, but instead have followed the practice which Hilditch (1947) applied to complex mixtures of unsaturated acids of the same number of C atoms, and have expressed the mean unsaturation in terms of H.

In the usual ester-fractionation technique it is not feasible to resolve the trace amounts of higher saturated and unsaturated acids. It now appears that in butterfat there is present a complex mixture of C_{20} and C_{22} unsaturated acids (Shorland & Johannesson, 1951), while the saturated acids above C_{18}, according to Helz & Bosworth (1936), are mainly C_{26}, with traces of C_{20}, C_{22} and C_{24} acids.

In determining the proportions of unsaturated acids, of equivalent greater than that of the C_{18} unsaturated components, the error was minimized by refractionation of the residue, the final residual fraction (approx. 2% of total fatty acids) containing little other than C_{20} or higher unsaturated acids, together with polymerized material. Hilditch (1947) calculated the butterfat saturated acids above C_{18} as C_{20}, but for the unsaturated acids above C_{18} he assumes a mean equivalent of 330 and describes these acids as C_{20}–C_{22}. This description is no doubt correct, but as the proportions of C_{20} and C_{22} components are unknown, there is the difficulty of assigning an exact value for the saponification equivalent. Moreover, recent investigations have established the presence of trace amounts of substances hitherto unidentified in butterfat. In this category are included certain C_{20} unsaturated acids (Shorland & Johannesson, 1951) and the various branched-chain acids (Hansen & Shorland, 1951 *a*, *b*; Hansen, Shorland & Cooke, 1951). One of these side-chain acids, incidentally, is a multi-branched C_{20} saturated acid. The determination of all these minor constituents is beyond the scope of present-day techniques of ester fractionation. Accordingly, for simplicity in both the saturated and unsaturated series, the acids above C_{18} were assumed to be entirely C_{20}.

In calculating those 'solid' fractions with saponification equivalents between 268 and 296, it was assumed that any unsaturation present was due to traces of methyl oleate, and that separation into 'solids' and 'liquids' by crystallization from acetone at $-30°$ had removed from the 'solids' all the C_{16} unsaturated acids (cf. Hilditch, 1947). This assumption was based on the following evidence:

(*a*) The solubility curves of Foreman & Brown (1944) exclude hexadecenoic acid from the 'solids'.

(*b*) Only minor proportions of hexadecenoic acid (about 2%) are present in butterfat.

(*c*) The crystallization was carried out with precision and the proportions of unsaturated acids in the 'solids' (iodine value approx. 3·0) was low and in the fractions of molecular

weight approximating 270, almost negligible (iodine value approx. 0·3).

In resolving the steam-volatile fractions into their component fatty acids it was assumed that unsaturation when present in the fractions of lower boiling point was due to decenoic acid (C_{10}) with an iodine value of 149·6. No unsaturated acid lower in the series than C_{10} has been isolated from butterfat. Investigation of the steam-volatile residues showed that they contained unsaturated components of molecular weight greater than that of decenoic acid. As the C_{18} unsaturated acid components constitute up to 85% of the total unsaturated acids of butterfat, the practice in these analyses was to assume this higher molecular weight unsaturated material in the residues from the steam-volatile acids to be C_{18}, its presence probably being due to the slight steam-volatility of oleic acid and possibly to 'splashing' during steam distillation.

The presence of oleic acid in the volatile residues was further indicated when a methyl ester sample of this material, on repeated crystallization from acetone and from methanol, yielded a fraction with saponification equivalent 291·2 and iodine value 79·2.

DISCUSSION

In Table 1 are presented the mean values for triplicate fatty acid composition analyses of six butterfat samples churned at approximately two-monthly intervals over the period of 1 year. Accompanying these results is the standard deviation of each constituent both within analyses and overall analyses. It will be noted from Table 1 that five of the samples with churning dates 15. ix. 47, 18. xi. 47, 28. i. 48, 6. iv. 48 and 18. v. 48 are representative of one dairying season, which in New Zealand commences about mid-July. The sixth sample was churned 28. vii. 48, which date, under normal conditions, corresponds approximately with the beginning of a new season. Evidence to be presented later in this paper indicates that only minor differences exist in the fatty acid composition of butterfats derived in the same months of different years. Therefore, in order to compare a series of analyses typical of any one complete season, the July sample of 28. vii. 48 (D/34) is assumed to correspond with the July sample of a year earlier. The six samples in Table 1 are then arranged in the order of a season which commences in July and ends in May.

Seasonal variations in the proportions of the fatty acid constituents

In comparing the analyses of six butterfat samples shown in Tables 1 and 2, it will be observed that the most characteristic feature of this series of analyses is the marked change in chemical composition which occurs when the season has advanced 3–4 months. This is well illustrated in the uniform pattern of changes followed by each member of the C_6–C_{14} group of saturated acids, and also by the C_{18} group of

Table 1. *Triplicate fatty acid composition analyses of six butterfats churned at approximately two-monthly intervals over the period of a year*

(Mean values (moles %) and standard deviation of each constituent within analyses and overall analyses are recorded.)

Sample	Date of churning	Saturated fatty acids									Unsaturated fatty acid						Total saturated acids	C_4–C_{12} saturated acids
		C_4	C_6	C_8	C_{10}	C_{12}	C_{14}	C_{16}	C_{18}	C_{20}	C_{10}	C_{12}	C_{14}	C_{16}	C_{18}	C_{20}		
D/34	28. vii. 48	12·0 ±0·23	3·7 ±0·06	0·9 ±0·21	2·7 ±0·17	2·9 ±0·25	8·4 ±0·21	23·6 ±0·15	11·3 ±0·25	0·4 ±0·20	0·2 ±0·06	0·2 ±0·00	0·9 ±0·12	2·4 ±0·12	29·5 ±0·25	0·9 ±0·00	65·9 ±0·23	22·2 ±0·12
C/38	15. ix. 47	11·5 ±0·21	4·5 ±0·06	1·5 ±0·30	3·5 ±0·20	3·5 ±0·17	9·6 ±0·12	21·6 ±0·12	11·3 ±0·06	0·7 ±0·10	0·2 ±0·00	0·2 ±0·00	0·8 ±0·10	1·8 ±0·12	28·1 ±0·31	1·3 ±0·00	67·6 ±0·17	24·4 ±0·29
C/51	18. xi. 47	10·8 ±0·17	4·7 ±0·15	1·9 ±0·25	4·4 ±0·20	4·4 ±0·20	11·5 ±0·21	22·9 ±0·31	11·1 ±0·10	0·6 ±0·12	0·4 ±0·00	0·3 ±0·06	1·0 ±0·00	1·7 ±0·06	23·4 ±0·12	1·2 ±0·12	72·1 ±0·20	26·1 ±0·42
C/56	28. i. 48	11·4 ±0·35	4·3 ±0·06	1·4 ±0·32	3·6 ±0·12	3·7 ±0·15	10·6 ±0·32	23·1 ±0·10	11·2 ±0·25	0·7 ±0·10	0·3 ±0·06	0·3 ±0·06	1·0 ±0·06	1·7 ±0·21	25·6 ±0·21	1·1 ±0·10	70·0 ±0·17	24·4 ±0·59
D/3	6. iv. 48	9·6 ±0·23	3·9 ±0·20	1·2 ±0·20	3·0 ±0·21	3·1 ±0·32	10·4 ±0·31	22·0 ±0·10	11·9 ±0·10	0·6 ±0·12	0·2 ±0·06	0·3 ±0·10	1·2 ±0·12	2·0 ±0·10	29·2 ±0·17	1·4 ±0·15	65·7 ±0·23	20·7 ±0·10
D/12	18. v. 48	9·5 ±0·21	3·5 ±0·06	1·2 ±0·17	2·8 ±0·29	2·9 ±0·15	9·7 ±0·05	22·7 ±0·17	12·2 ±0·12	0·8 ±0·12	0·2 ±0·00	0·2 ±0·06	1·1 ±0·06	2·0 ±0·12	29·8 ±0·00	1·4 ±0·00	65·3 ±0·15	19·8 ±0·21
Overall standard deviation		±0·24	±0·11	±0·25	±0·20	±0·22	±0·22	±0·17	±0·16	±0·13	±0·04	±0·05	±0·08	±0·13	±0·25	±0·11	±0·20	±0·33

unsaturated acids. These two groups of constituents comprise approximately 50 moles % of the total fatty acids. At the beginning of lactation the C_6–C_{14} acids comprise 18·6 %, and the C_{18} unsaturated acids 29·5 % of the total molar content, but these proportions change to 26·9 and 23·4 % respectively in November, and then gradually return, at the end of lactation, to proportions similar to those at the beginning of the season. Although these changes in fatty acid composition involve a general compensation between the C_6–C_{14} acids and the C_{18} unsaturated acids, the balance is not precise. For example, in July the low value for the C_6–C_{14} saturated acids (18·6 moles %) is compensated for by high values in the C_4 and C_{16} saturated acids respectively, as well as by high values in the C_{18} unsaturated acids. In May, however, the low value for the C_6–C_{14} saturated acids (20·1 moles %) is compensated for by a high content of stearic acid and of C_{18} unsaturated acids, the butyric acid content being at a minimum. The amount of butyric acid tends to decrease with the advance of the season, but its variations at two-monthly intervals are irregular. Most marked of the differences in C_4 content is that between the July sample (12·0 moles %) and the May sample (9·5 moles %). When, however, the butyric acid content is expressed on a weight percentage basis (cf. Table 2) a more uniform trend is illustrated and the decline in content is shown to range from 4·5 % in July to 3·5 % in May.

Palmitic acid, the major saturated component, is shown in Table 1 to be relatively constant in amount throughout the season, the highest value of 23·6 moles % occurring at the beginning of the season in July, and the lowest value of 21·6 moles % appearing less than 2 months later in September. Whereas the C_4–C_{14} saturated acid components are characterized by a decrease in content from November to the end of the season, stearic acid is unique in that it shows a small but regular increase during this period. The total saturated acids in each of the six butterfat samples follow the same pattern of variation as the individual C_6–C_{14} saturated acids. Statistical comparison of these latter constituents reveals the differences to be significant at the 1 % level in twelve of the fifteen comparisons (cf. Table 4). When the component saturated acids are expressed as a percentage of the total saturated acids in each sample (see Table 3), they display, unlike the unsaturated acids when similarly expressed, appreciable variations. Palmitic acid, for example, ranges from 35·8 moles % in July to 31·7 moles % in November. Butyric acid changes erratically when expressed on this basis, while the C_6–C_{14} saturated components show a regular movement throughout the season, the contents rising to a maximum in November, and then slowly declining

Table 2. *Physical and chemical data for six butterfats representing churnings made at approximately two-monthly intervals over the period of a year*

Butterfat sample	Date of churning	Iodine value (Wijs)	C_{18} unsaturated acids (moles %)	Saponification equiv.	Saponification value (calc.)	Reichert value*	C_4–C_{12} saturated acids (moles %)	Softening point†	C_4 acids (wt. %)	Total acids liquid at room temp. (moles %)	Total acids liquid at room temp. (wt. %)
D/34	28. vii. 48	39·9	29·5	247·8	226·4	31·1	22·2	32·7	4·5	50·7	47·3
C/38	15. ix. 47	38·4	28·1	244·4	229·6	31·7	24·4	32·3	4·3	49·9	47·4
C/51	18. xi. 47	33·6	23·4	240·5	233·3	32·0	26·1	32·7	4·2	45·4	46·4
C/56	28. i. 48	36·8	25·6	244·0	229·9	30·1	24·4	33·4	4·4	47·1	43·6
D/3	6. iv. 48	39·9	29·2	249·1	225·3	27·3	20·7	33·7	3·5	49·0	41·4
D/12	18. v. 48	40·8	29·8	249·3	225·1	25·2	19·8	34·3	3·5	48·9	46·3

* Determinations made by Dairy Research Institute, Palmerston North, New Zealand. (In some cases Reichert values were made on samples churned within a day or two of those reported here.)
† Softening points determined by Dairy Research Institute, Palmerston North, New Zealand, using the method of Barnicoat (1944).

Table 3. *Saturated acid components as percentage of total saturated acids, and unsaturated acid components as percentage of total unsaturated acids*

	Saturated acid components expressed as percentage of total saturated acids									Unsaturated acid components expressed as percentage of total unsaturated acids					
Sample	C_4	C_6	C_8	C_{10}	C_{12}	C_{14}	C_{16}	C_{18}	C_{20}	C_{10}	C_{12}	C_{14}	C_{16}	C_{18}	C_{20}
D/34	18·2	5·6	1·4	4·1	4·4	12·7	35·8	17·2	0·6	0·6	0·6	2·6	7·0	86·6	2·6
C/38	17·0	6·6	2·2	5·2	5·2	14·2	31·9	16·7	1·0	0·6	0·6	2·5	5·6	86·7	4·0
C/51	14·9	6·5	2·6	6·1	6·1	15·9	31·7	15·4	0·8	1·4	1·0	3·6	6·0	83·8	4·2
C/56	16·3	6·1	2·0	5·1	5·3	15·2	33·0	16·0	1·0	1·0	1·0	3·3	5·7	85·3	3·7
D/3	14·6	5·9	1·8	4·6	4·7	15·9	33·5	18·1	0·9	0·6	0·9	3·5	5·8	85·1	4·1
D/12	14·5	5·4	1·8	4·3	4·4	14·9	34·8	18·7	1·2	0·6	0·6	3·2	5·8	85·8	4·0

Table 4. *Significant differences between sample means on the basis of the 't' test, and the classification of pairs of samples whose differences are significant at 5 and 1 % level*

(Designation of samples: a, C/38 (churned 15. ix. 47); b, C/51 (churned 18. xi. 47); c, C/56 (churned 28. i. 48); d, D/3 (churned 6. iv. 48); e, D/12 (churned 18. v. 48); f, D/34 (churned 28. vii. 48).)

	Saturated fatty acids									Unsaturated fatty acids						Total saturated	C_4–C_{12}
	C_4	C_6	C_8	C_{10}	C_{12}	C_{14}	C_{16}	C_{18}	C_{20}	C_{10}	C_{12}	C_{14}	C_{16}	C_{18}	C_{20}		
5% significant difference	0·43	0·20	0·44	0·36	0·39	0·40	0·31	0·29	0·23	0·07	0·08	0·15	0·23	0·44	0·19	0·59	0·35
1% significant difference	0·60	0·28	0·62	0·51	0·54	0·56	0·43	0·41	0·32	0·10	0·12	0·20	0·32	0·61	0·27	0·83	0·49
Not significant	ac de	ab df	ab de ac df ad ef ae bc cd ce	ac de df ef	ac de df ef	ae cd	bc be ce	ab ac af bc bf cf	ab bf ac cd ad ce ac de bc bd	ad ef ae af bc de df	af de bc ef bd cd ce cf	af cf bc de be ef bf cd ce	ab ac ae bc de	df ef	ab ad ae bc de	de df	ac
Significant at 5% but not at 1 %	af cf	ac	cf	—	ad	—	ad	de	af df be cf	cd	ae be df	ab bd	ad ce be cd	de	ae bf cf	ef	—
Significant at 1%	ab cd ad ce ae df bc ef bd be bf	ad cd ae ce af cf bc de bd ef be bf	af bd be bf	ab bf ad cd ae ce af cf bc bd be	ab cd ae ce af cf bc bd be bf	ab bf ac ce ad cf af de bc df bd cf be	ab cf ac de ae df af ef bd bf cd	ad ef ae bd be cd ce df	ef	ab cf ac bd be bf ce	ab ac ad bf	ac ad ae df	af bd bf cf df ef	ab bd ae be ad bf ae cd af ce bc cf	af ef bd be cd ce df	ab be ac bf ae ce ad cd af cf bc bd	ab be ad bf ae cd af ce bc cf bd de df ef

to a minimum in May. The C_6–C_{14} saturated acids follow the same trends as they do when expressed as percentages of the total fatty acids.

Of the six butterfat analyses shown in Table 1, those which exhibit extreme differences are the November (C/51) and the May (D/12) samples. Statistical interpretation of the variations between these samples demonstrates significant differences between all components, except the C_{14} unsaturated acids which are present in minor amounts. Of these differences, ten of the thirteen are significant at the 1 % level (cf. Table 4). The C_{18} unsaturated acids (the major constituents of butterfat) are at a minimum in November (23·4 moles %) and rise to a maximum in May (29·6 moles %), a seasonal variation of 6·2 moles %. The accentuated difference between these two samples does, however, conform to the findings of Cox & McDowall (1948), who observed that seasonal changes in New Zealand butterfats were characterized by low average iodine values in November–December (about 34·0) which rose to relatively high values in May–June (about 40·0).

While the content of C_{18} unsaturated acids in New Zealand butterfats is limited to the range of 23·4–29·8 moles % the figures presented by Hilditch & Jasperson (1941) for English butterfats vary from 25·5 to 37·7 moles %. The average of five values cited by Hilditch (1947) was 34·8 moles %. For a sample of Californian butterfat, Jack & Henderson (1945) recorded the surprisingly low figure of 17·0 moles %.

The palmitic acid content of New Zealand butterfat is relatively constant (21·6–23·6 moles %) while that of English butterfat is shown to range between 21·1 moles % (Hilditch & Paul, 1940) and 26·7 moles % (Hilditch & Jasperson, 1941). Jack & Henderson (1945) report 27·2 moles % of palmitic acid in their Californian sample, and Achaya & Banerjee (1945) show samples of Indian buffalo ghees as fluctuating from 19·0 to 31·9 moles % of this constituent. From the evidence available, it would appear that New Zealand butterfats, by comparison with English butterfats, are characterized generally by a lower content of C_{18} unsaturated acids and of palmitic acid.

Annual variations in fatty acid composition

In Table 5 are recorded the results of triplicate analyses of a further four butterfat samples. For the purpose of determining annual variations in fatty acid composition, data are shown in Table 6 representing the differences between means of analyses of butterfats churned in November 1946 (B/36) and November 1947 (C/51), and between May 1946 (C/18) and May 1947 (D/12) respectively, together with the levels at which these differences are significant.

Table 5. *Triplicate fatty acid composition analyses of four butterfat samples*

Sample	Date of churning	Saturated fatty acid								Unsaturated fatty acid					Total saturated	Total unsaturated
		C_4	C_6	C_8	C_{10}	C_{12}	C_{14}	C_{16}	C_{18}	C_{20}	C_{10}	C_{12}	C_{14}	C_{16}	C_{18}	C_{20}		
B/1, 9	About	10·0	4·2	1·7	3·3	3·6	10·3	23·7	10·7	0·6	0·3	0·3	1·4	2·3	26·1	1·5	68·1	31·9
B/1, 12	5. iv. 45	10·8	3·6	1·8	3·3	4·0	9·5	22·6	11·4	0·5	0·3	0·3	1·2	1·7	27·2	1·8	67·5	32·5
B/1, 25		10·8	3·8	2·0	3·3	3·7	9·5	23·6	10·9	0·6	0·3	0·3	1·1	1·7	27·0	1·4	68·2	31·8
	Mean	**10·5**	**3·9**	**1·8**	**3·3**	**3·8**	**9·8**	**23·3**	**11·0**	**0·6**	**0·3**	**0·3**	**1·2**	**1·9**	**26·7**	**1·6**	**68·0**	**32·0**
B/36, 1	About	10·7	5·0	2·2	4·2	5·4	12·3	23·0	9·9	0·6	0·3	0·3	1·0	1·5	21·7	1·9	73·3	26·7
B/36, 6	18. xi. 46	11·3	4·5	2·2	4·6	4·8	11·8	22·9	10·7	0·6	0·3	0·3	1·0	1·5	22·2	1·3	73·4	26·6
B/36, 10		10·6	4·8	2·2	4·2	5·2	12·6	22·6	10·3	0·6	0·3	0·3	1·1	1·8	22·2	1·2	73·1	26·9
	Mean	**10·9**	**4·8**	**2·2**	**4·3**	**5·1**	**12·2**	**22·8**	**10·3**	**0·6**	**0·3**	**0·3**	**1·0**	**1·6**	**22·1**	**1·5**	**73·2**	**26·8**
C/18, 1	About	10·3	3·6	1·4	2·8	3·3	9·3	21·9	11·8	0·6	0·3	0·3	0·9	1·9	29·5	2·1	65·0	35·0
C/18, 2	13. v. 47	10·3	3·8	1·4	2·6	3·6	9·3	21·4	11·7	0·9	0·3	0·3	0·9	1·8	29·6	2·1	65·0	35·0
C/18, 3		10·0	4·1	1·1	2·7	3·2	9·6	21·6	11·8	0·5	0·2	0·2	1·0	2·1	29·8	2·1	64·6	35·4
	Mean	**10·2**	**3·8**	**1·3**	**2·7**	**3·4**	**9·4**	**21·6**	**11·7**	**0·7**	**0·3**	**0·3**	**1·0**	**1·9**	**29·6**	**2·1**	**64·8**	**35·2**
D/86, 1	14. iii. 49	10·5	3·9	1·1	3·4	3·8	9·9	23·2	11·9	0·4	0·3	0·3	1·0	1·6	27·0	1·7	68·1	31·9
D/86, 3		10·6	4·2	1·2	3·0	3·5	9·4	23·7	11·6	0·6	0·3	0·3	1·2	1·8	27·1	1·5	67·8	32·2
D/86, 4		11·0	4·2	1·2	2·8	3·5	9·7	23·3	11·5	0·4	0·3	0·3	1·1	1·9	26·9	1·9	67·6	32·4
	Mean	**10·7**	**4·1**	**1·2**	**3·0**	**3·6**	**9·6**	**23·4**	**11·7**	**0·5**	**0·3**	**0·3**	**1·1**	**1·8**	**27·0**	**1·7**	**67·8**	**32·2**

Statistical comparison of the two November samples B/36 and C/51 (cf. Table 6), shows that the variables of greatest magnitude are the C_{12}, C_{14} and C_{18} saturated acids, and the C_{18} unsaturated acids. All of these differences are significant at the 1% level. In the two May samples C/18 and D/12, the variables significant at the 1% level are the saturated acids C_4 and C_{16}, and the C_{20} unsaturated acids, which latter are present in minor amounts. It is not surprising that the C_{18} unsaturated acids show significant differences in the November samples, for these acids undergo rapid changes in content at this time of the year (cf. Table 1) and it would be considered unlikely that the period of minimum content of this constituent would fall at precisely the same time each year. With this reservation it is considered that the statistical evaluation of the differences in fatty acid composition of butterfats churned in corresponding months of different years indicates that most of the components do not fluctuate appreciably in amount, and that where significant differences do occur the percentage variations are relatively small.

Although the two comparisons referred to distinguish between fatty acid composition of two examples only, both of which represent samples churned in corresponding months of consecutive years, nevertheless, the results serve as a guide to the probable limits of annual variation.

Table 6. *Differences between means of samples taken at corresponding months of different years, and the level at which they are significant*

Samples	Saturated fatty acids (moles %)									Unsaturated fatty acids (moles %)						
	C_4	C_6	C_8	C_{10}	C_{12}	C_{14}	C_{16}	C_{15}	C_{20}	C_{10}	C_{12}	C_{14}	C_{16}	C_{18}	C_{20}
B/36 (churned 18. xi. 46) – C/51 (churned 18. xi. 47)	+0·07	+0·10	+0·33	−0·07	+0·73	+0·76	0·00	−0·80	+0·03	−0·10	−0·03	+0·03	−0·07	−1·34	+0·34
Level of significance	—	—	—	—	1%	1%	—	1%	—	1%	—	—	—	1%	5%
C/18 (churned 18. v. 47) – D/12 (churned 18. v. 48)	+0·73	+0·36	+0·10	−0·07	+0·44	−0·33	−1·07	−0·46	−0·16	+0·07	+0·04	−0·10	−0·04	−0·17	+0·67
Level of significance	1%	5%	—	—	5%	—	1%	5%	—	5%	—	—	—	—	1%

Relationship between chemical and physical constants

As shown in Table 2, both the July and the September butterfats (D/34 and C/38) possess high iodine values of 39·9 and 38·4 respectively, while the November sample displays a sudden drop to a minimum value of 33·6, which slowly rises to give a maximum value of 40·8 in May (D/12). The corresponding C_{18} unsaturated acids follow the same trend. Although the iodine values are not directly proportional to the C_{18} unsaturated acid contents, the figures suggest that the iodine values are an indication of the amount of these acids present (cf. Bartley *et al.* 1951).

For the six butterfats considered in Table 1, the sum of the C_{18} unsaturated acids and the total saturated acids in each sample is constant within very narrow limits (95·6, 95·4, 94·9, 95·7, 95·6 and 95·5%) and constitutes an average of 95·5% of the total molar weight. Accordingly, values for the total saturated acids shown in Table 1 are as would be predicted from a knowledge of the C_{18} unsaturated content of corresponding samples. As the iodine value is an index of the C_{18} unsaturated acid content of butterfat, so also is it an index of the total content of saturated acids present. When the unsaturated acid components are expressed as a percentage of the total unsaturated acids, it is seen from Table 3

that the content of C_{18} unsaturated acids remains approximately constant over the period of a year, the values being 86·6 (D/34), 86·7 (C/38), 83·8 (C/51), 85·3 (C/56), 85·1 (D/3) and 85·8 % (D/12). Thus, although the total amount of unsaturated acids will vary at intervals, the C_{18} unsaturated acid components constitute an approximately fixed proportion of those totals.

The saponification equivalents and iodine values, without bearing any direct mathematical relationship with each other, follow the same trends. In conformity with the graphs of Cox & McDowall (1948) saponification equivalents when converted to saponification values exhibit an interrelationship with the Reichert values, an increase in one corresponding with an increase in the other. As might be anticipated, the Reichert values are closely correlated with the proportions of C_4–C_{12} saturated acids found to be present in the samples analysed, and are at a maximum of 32·0 in November and a minimum of 25·5 in May. In Table 2 it is shown that minimum values for both the saponification equivalents and the Reichert values occur in the May sample in which the content of C_{18} unsaturated acids is highest, and maximum values occur in the November sample in which the C_{18} unsaturated acid content is lowest. Thus changes in content of C_4–C_{12} saturated acids are reflected in compensating changes in the content of C_{18} unsaturated acids.

No correlation can be established between the softening points and iodine values of the butterfat samples reported in this paper. It appears, however, that softening points are influenced more by the content of lower saturated acids (C_4–C_{12}) than by the unsaturated components; high softening points corresponding with low contents of C_4–C_{12} saturated acids and also with low Reichert values (cf. Eckles & Palmer, 1916). Attempts to correlate softening points with the total content of acids liquid at room temperature (C_4, C_6 and C_8 saturated acids, together with all the unsaturated acids) have not established any definite relationship. From data in Table 2 it might be expected that the November sample C/51 with a content of 45·4 moles % of acids liquid at room temperature (minimum value recorded for the six samples) would possess a softening point higher than that of the other butterfats: actually its softening point is only 32·7°, which is identical with that of sample D/34 (July) with 50·7 moles % (maximum value recorded for these six samples). Conversely, if a definite relationship existed it would be expected that the May sample (D/12) with the highest softening point of 34·3° would possess the minimum value for the total acids liquid at room temperature. However, its content of liquid acids is 48·9 moles %, only 1 mole % lower than that for the September sample (C/38), which, of the six butterfats analysed, has the lowest softening point of 32·3°. A similar lack of interrelationship exists when the acids liquid at room temperature are expressed in terms of weight per cent (cf. Table 2). The absence of correlation between the content of acids liquid at room temperature and the softening points of corresponding samples, suggests that softening points depend on the mode of fatty acid distribution throughout the glyceride molecules.

Factors influencing seasonal variations in fatty acid composition of New Zealand butterfat

Among the many factors which have been suggested as being responsible for seasonal variations in the fatty acid composition of butterfat are (*a*) diet, (*b*) plane of nutrition, (*c*) stage of lactation, (*d*) temperature of environment, (*e*) degree of freedom of movement, (*f*) age of cow, and (*g*) breed of cow.

Diet. The influence of diet on the fatty acid composition of butterfat has been established by a number of experiments. Hilditch & Thompson (1936) fed linseed oil, which is rich in diene and triene C_{18} acids, and observed that there was no perceptible increase in the amounts of these substances present in the milk fats. When, however, cows were fed liberal amounts of palm-kernel oil (Hilditch & Jasperson, 1943), or coconut oil (Hilditch & Sleightholme, 1930), both of which substances are rich in lauric and myristic acids, the proportions of lauric and particularly of myristic acids in the butterfat were increased. Similarly, the feeding of rape-seed oil (Hilditch & Thompson, 1936), which contains erucic acid, led to the appearance of this acid in butterfat. The fats present in grass do not amount to more than 2–4 % of the dry matter (cf. Shorland, 1944), and their main constituents, linolenic and octadecadienoic acids, are found in not more than minor amounts (about 1 %) in butterfat (cf. Shorland, 1950). From the ample evidence available it appears that under normal grazing conditions the influence of diet would remain substantially the same throughout the season and would not be responsible for variations in fatty acid composition of butterfat.

Of the other factors referred to, those considered to have exerted greatest influence on the samples described in this paper are the plane of nutrition and the stage of lactation.

Plane of nutrition. Investigations by Eckles & Palmer (1916), Riddet (1938), Riddet, Campbell, McDowall & Cox (1941) and by Smith & Dastur (1938) have all established that underfeeding has marked effects on the physical and chemical constants of butterfat. Smith & Dastur (1938), who supported their evidence with fatty acid composi-

tion analyses, showed that inanition led to an increased oleic acid content accompanied by a reduction in the lower saturated acids. Their fatty acid composition analyses were consistent with the results of the other investigators who observed a rise in iodine value and a decrease in Reichert value with a lowering of the plane of nutrition.

In New Zealand, cows are pasture-grazed throughout the year, the diet normally being supplemented with hay or silage during winter. Growth of pasture reaches its maximum within the months of September to January and declines slowly towards the end of the season (May–June), the amount of feed available to cows in New Zealand diminishing accordingly. If the plane of nutrition were to influence the fatty acid composition of the butterfat samples shown in Table 1 it would be anticipated that when pasture was abundant, as it normally is in November, analyses would reveal a low content of C_{18} unsaturated acids, with a corresponding high content of lower volatile acids, and that as the season advanced and food became increasingly scarce, the content of C_{18} unsaturated acids would rise. This pattern has been followed, the minimum content of C_{18} unsaturated acids appearing in November (C/51) and the peak value in May (D/34). Similarly, the lower volatile acids (C_4–C_{12}) suffer a decline from 26·1 moles % in November to 19·8 moles % in June.

In Great Britain when the cows change over from stall-feeding to pasture-feeding in the summer the animals suffer a decline in the plane of nutrition and the iodine value rises to a maximum. It would appear that this factor may be responsible for the differences in composition observed between New Zealand and English summer butterfat. The analyses by Hilditch & Jasperson (1941) for English summer butterfat from pasture-fed animals are in close agreement with our analyses for New Zealand winter butterfat, except that the English butterfat characteristically has a slightly higher content of C_{18} unsaturated acids. Similarly, Hilditch & Sleightholme (1930) observed a close relationship between New Zealand summer butterfat and an English (stall-fed) winter butterfat.

Stage of lactation. That the stage of lactation exerts an effect upon the composition of butterfat was reported by Eckles & Shaw (1913), Holland & Buckley (1918), and by Riddet *et al.* (1941). The former observed that the volatile acids showed a uniform decline from the beginning to the end of the lactation period, and that the iodine value underwent a gradual increase, the highest point being reached at the end of the season. Similar trends in iodine values which appeared, regardless of seasonal changes in temperature, and in which the variations in iodine value were associated largely with changes in the estimated oleic acid content of the fat, have been reported by Bartley *et al.* (1951). Hilditch & Sleightholme (1930), when commenting on the milk-fat analyses published by Holland & Buckley (1918), also considered that the amount of unsaturated acids in butter increased with the time the cows had been in lactation.

In this present study of New Zealand butterfats, the contents of C_{18} unsaturated acids (which have been shown to be closely correlated with iodine values) do not gradually increase throughout the season as anticipated from the investigations of Eckles & Shaw (1913), but rather do they follow the trends of Bartley *et al.* (1951), who observed that milk fats possessed a high iodine value during the early stages of lactation followed by a decline (about 8 units of iodine value) to the fourth or fifth month of lactation, and then a slight increase (about 3 units of iodine value) to the end of lactation. Samples analysed in this present survey decreased in content of C_{18} unsaturated acids from 29·5 moles % at the commencement of lactation in July, to 23·4 moles % in November, and only after that date did the content of these acids increase as the season advanced, reaching a peak (29·8 moles %) at the end of lactation. Whereas Bartley *et al.* (1951) found that the iodine value of the butterfat was 5 units lower at the end of the lactation period than at the beginning, our results show that the New Zealand butterfats produced at the beginning and at the end of the lactation period have approximately the same iodine values.

The figures reported in Tables 1 and 2 are consistent with the changes in fatty acid composition resulting from the varying planes of nutrition throughout the season. Furthermore, they conform to those seasonal trends in content of C_{18} unsaturated acids observed by Bartley *et al.* (1951) to accompany the advance of lactation.

From the data now presented for New Zealand butterfat, it is felt that it is not possible to differentiate between the effects of the plane of nutrition and the stage of lactation. It is anticipated, however, that this problem will be elucidated by experiments now being conducted by the Dairy Research Institute, Palmerston North, New Zealand.

In conclusion, it is considered that the results presented in this paper illustrate the trends of the variations in content of each of the major fatty acid constituents of New Zealand butterfat during one season. The analyses further indicate that only minor changes occur in the composition of butterfat samples in the same month of different years. This information, when supported by the comprehensive data relating to the constants of butterfat over a 4-year period (Cox & McDowall, 1948), affords a guide to the seasonal changes that can be predicted in the fatty acid composition of New Zealand butterfat.

SUMMARY

1. Six butterfats taken at approximately two-monthly intervals throughout the season have been subjected to ester-fractionation analyses in triplicate and the standard deviation for each constituent has been calculated.

2. Throughout the season, beginning in July, there was found to be a regular increase in the content of C_6–C_{14} saturated acids (and total saturated acids) up to November, and thereafter a slow decline to the end of the season. The C_{18} unsaturated acids varied in the reverse direction to that of the C_6–C_{14} constituents.

The butyric acid content changed irregularly, but tended to decline throughout the season. Palmitic acid showed little change, but when expressed as a percentage of the total saturated acids it showed a tendency to increase throughout the season.

3. In comparing butterfats sampled at two-monthly intervals during 1 year, statistical evaluation showed that significant differences existed between many of the fatty acid constituents. Extreme differences in composition were exhibited by the May and November samples, where, of the thirteen differences, ten were assessed to be significant at the 1 % level.

4. Comparing the butterfats taken during the same month, but in different years, significant differences were observed between certain of the component fatty acids. However, the differences were small, and this fact, together with Cox & McDowall's (1948) results showing the similarity between the constants of butterfats sampled during the same months of different years, is sufficient to indicate that the present study would serve as a useful guide for predicting the fatty acid composition of New Zealand butterfat for any given month.

5. The variations in content of C_{18} unsaturated acids reflect the influence of the changing plane of nutrition throughout the year and at the same time are consistent with the changes observed by Bartley *et al.* (1951) to accompany the progression of lactation.

The authors wish to acknowledge with appreciation the assistance in experimental work provided by Miss M. D. L. White.

To Dr F. H. McDowall, Chief Chemist, Dairy Research Institute, Palmerston North, New Zealand, we are indebted for arranging all butterfat samples used in this survey and for helpful discussions.

Statistical interpretations of the results reported in this paper were made by the Applied Mathematics Laboratory, Department of Scientific and Industrial Research, Wellington.

REFERENCES

Achaya, K. T. & Banerjee B. N. (1945). *Indian J. vet. Sci.* **15**, 271.

Barnicoat, C. R. (1944). *Analyst*, **69**, 176.

Bartley, E. E., Zaletel, J. H., Bird, E. W., Cannon, C. Y., Wise, G. H. & Kempthorne, O. (1951). *J. Dairy Sci.* **34**, 536.

Coulson, E. A. & Herington, E. F. G. (1948). *Trans. Faraday Soc.* **44**, 629.

Cox, G. A. & McDowall, F. H. (1948). *J. Dairy Res.* **15**, 377.

Eckles, C. H. & Palmer, L. S. (1916). *Res. Bull. Mo. agric. Exp. Sta.* no. 25.

Eckles, C. H. & Shaw, R. H. (1913). *The Influence of the Stage of Lactation on the Composition and Properties of Milk.* U.S. Dep. of Agriculture.

Foreman, H. D. & Brown, J. B. (1944). *Oil & Soap*, **21**, 183.

Hansen, R. P. (1951). *J. Amer. Oil Chem. Soc.* **28**, 375.

Hansen, R. P. & Shorland, F. B. (1951). *Biochem. J.* **50**, 207.

Hansen, R. P. & Shorland, F. B. (1952). *Biochem. J.* **50**, 358.

Hansen, R. P., Shorland, F. B. & Cooke, N. J. (1951). *Chem. & Ind.* p. 839.

Helz, G. E. & Bosworth, A. W. (1936). *J. biol. Chem.* **116**, 203.

Hilditch, T. P. (1947). *The Chemical Constitution of Natural Fats*, 2nd ed. revised. London: Chapman and Hall.

Hilditch, T. P. & Jasperson, H. (1941). *J. Soc. chem. Ind., Lond.*, **60**, 305.

Hilditch, T. P. & Jasperson, H. (1943). *Biochem. J.* **37**, 238.

Hilditch, T. P. & Jones, E. E. (1929). *Analyst*, **54**, 75.

Hilditch, T. P. & Paul, S. (1940). *J. Soc. chem. Ind.* **59**, 138.

Hilditch, T. P. & Sleightholme, J. J. (1930). *Biochem. J.* **24**, 1098.

Hilditch, T. P. & Thompson, H. M. (1936). *Biochem. J.* **30**, 677.

Holland, E. B. & Buckley, J. P. (1918). *J. agric. Res.* **12**, 719.

Jack, E. L. & Henderson, J. L. (1945). *J. Dairy Sci.* **28**, 65.

Jack, E. L., Henderson, J. L. & Hinshaw, E. B. (1946). *J. biol. Chem.* **162**, 119.

Longenecker, H. E. (1937). *J. Soc. chem. Ind.* **56**, 199T.

New Zealand Department of Agriculture (1948). *Primary Production in New Zealand.*

Riddet, W. (1938). *Proc. 7th. Ann. Conf. N.Z. Grasslands Ass.*

Riddet, W., Campbell, I. L., McDowall, F. H. & Cox, G. A. (1941). *N.Z. J. Sci. Tech.* **23**, 99A.

Shorland, F. B. (1944). *Nature, Lond.*, **153**, 168.

Shorland, F. B. (1950). *Nature, Lond.*, **166**, 745.

Shorland, F. B. & Johannesson, D. L. (1951). *Nature, Lond.*, **168**, 75.

Smith, J. A. B. & Dastur, N. N. (1938). *Biochem. J.* **32**, 1868.

The Biosynthesis of Protein

1. UPTAKE OF GLYCINE, SERINE, VALINE AND LYSINE BY THE MAMMARY GLAND OF THE RABBIT

By P. N. CAMPBELL and T. S. WORK
National Institute for Medical Research, The Ridgeway, Mill Hill, London, N.W. 7

(*Received* 10 *December* 1951)

Owing to the failure to detect in tissue extracts any peptides which might serve as intermediates in the synthesis of proteins from amino-acids, theories have been advanced for the synthesis of proteins in which intermediates between amino-acids and proteins are not involved. Most of these theories depend on some sort of template mechanism (see Haurowitz, 1950, for general review). However, the possibility that peptides may play a role in protein synthesis has been revived by the isolation in the past 10 years of many antibiotic peptides. Various explanations have been sought for the striking biological activity of these compounds (Hotchkiss, 1944; Work, 1949; Harris & Work, 1950). The hypothesis that these antibiotics might act by virtue of structural similarity to intermediates in protein synthesis prompted us to seek evidence for the occurrence of peptides as intermediates in protein synthesis (Campbell & Work, 1952).

Although the information in the literature on the biosynthesis of proteins and on the part played by peptide intermediates is decidedly conflicting, there is some evidence for the metabolic activity of peptides. Thus, although small peptides other than glutathione have not been isolated from fresh cell extracts, Borsook and his colleagues have shown that various cells contain peptide material of higher molecular weight. In a tissue homogenate, glycine and leucine are incorporated faster into this peptide than into proteins (Deasy, Borsook, Haagen-Smit, Keighley & Lowy, 1949). Fischer (1948) also has found that tissue cultures of chicken myoblasts grow much better on a partial hydrolysate of protein than on a complete hydrolysate.

Against this evidence in favour of the utilization of peptides must be set the failure of peptides obtained by partial hydrolysis of proteins to be utilized in intravenous infusion experiments in men and in dogs. Such mixtures were less effective in maintaining nitrogen balance than a complete hydrolysate of the same protein, and most of the peptide was excreted in the urine (Christensen, 1950; Silber & Porter, 1949).

Experiments on the turnover rate of isotopically labelled amino-acid in proteins have also been interpreted as favouring the exclusion of peptides as normal intermediates in protein synthesis. No difference has been found between the half times for the incorporation of various amino-acids into rat-liver proteins, and Rittenberg (1948), in discussing this result, points out that if peptides were intermediates each amino-acid would probably have a characteristic and different half time. Muir, Neuberger & Perrone (1951) came to the same conclusion after estimating the activity of the end-group valine and the residual valine of haemoglobin. The results of experiments involving passive immunity in rabbits favour the view that interconversion of one protein to another can only take place after complete hydrolysis of the protein to its constituent amino-acids and not through peptide intermediates (Heidelberger, Treffers, Schoenheimer, Ratner & Rittenberg, 1942; Rittenberg & Shemin, 1946; Bulman & Campbell, 1951).

If peptides were intermediates in protein synthesis we might expect to find evidence for their utilization as nutrients for micro-organisms, but synthetic peptides have been found to be inferior to, or no better than, the corresponding free amino-acids as nutrients for yeast (Danté & Thorne, 1949). However, Barton-Wright (1951) found peptone and wort superior sources of nitrogen for yeast. Various synthetic peptides have also been tried as growth factors for bacteria. In most cases the peptide appears to undergo hydrolysis before being utilized, but in a few cases the peptide may be used without prior degradation (Ågren, 1947; Simmonds & Fruton, 1948; Krehl & Fruton, 1948; Simmonds & Fruton, 1949*a*, *b*; Taylor, Simmonds & Fruton, 1950; Malin, Camien & Dunn, 1951; Nurmikko & Virtanen, 1951; Virtanen & Nurmikko, 1951). That partial hydrolysates of proteins have been found to have greater growth-promoting properties for bacteria than complete amino-acid mixtures (Sprince & Woolley, 1945; Dunn & McClure, 1950; Klungsøyr, Sirny & Elvehjem, 1951) also supports the hypothesis that micro-organisms can utilize peptides *per se*. These observations, together with our own studies on antibiotic peptides (Work, 1949; Harris & Work, 1950; Callow & Work, 1952), suggested to us that an important factor in the utilization of peptides might be amino-acid

sequence and that certain sequences might be characteristic for any one species. We decided, therefore, to examine the mechanism of synthesis of a particular group of proteins, the milk proteins; these are synthesized in the intact animal under conditions such that peptides produced by partial degradation of the animal's own plasma protein would have an excellent chance of being re-utilized for milk synthesis before degradation was complete.

Formation of milk protein was followed in lactating rabbits after injection of radioactive [^{14}C] glycine, valine and lysine respectively. The radioactivity of plasma protein was compared with that of milk protein and each of the three amino-acids was re-isolated from the milk protein and its specific radioactivity determined. In another experiment, radioactive plasma ([^{14}C] glycine) from one rabbit was injected into a second animal and the radioactivity of the milk and plasma compared after suitable intervals. It is tentatively concluded that the plasma protein may supply some peptides for milk synthesis, but that the larger proportion of the nitrogen of milk protein is derived from the free amino-acids of the blood. The data on specific radioactivities of amino-acids re-isolated from milk protein favour the idea that peptides are intermediates in protein synthesis.

A preliminary account of this work has already been published (Campbell & Work, 1951).

MATERIALS AND METHODS

Glycine. The amino-acid was obtained from the Radiochemical Centre, Amersham, as [α-^{14}C] glycine.

Valine. Valine was supplied by the Radiochemical Centre as DL-valine with ^{14}C in the *gem*-dimethyl groups.

Lysine. DL-Lysine labelled with ^{14}C in the α-position was kindly supplied by Dr H. R. V. Arnstein of this Institute (Arnstein, Hunter, Muir & Neuberger, 1952).

Animals. Rabbits were used. The animals were allowed to suckle their own litter (not less than four) for 2 weeks and were during that period handled frequently so as to accustom them to being milked. They were fed *ad lib.* with a standard pellet diet (Diet 18, Bruce & Parkes, 1946).

Milking. The litter was removed from the cage and the animal left undisturbed with food and water for about 5 hr. After this time an intravenous injection of 2 i.u. posterior pituitary extract (British Drug Houses, Ltd.) was given and milking was started at once, using a rubber catheter to fit over the nipple and applying an intermittent suction of 10–20 cm. mercury. When necessary, a second injection of hormone was given after half an hour. When enough milk had been obtained, the animal was returned to its litter and left with it until 5 or 6 hr. before the next milking. It was found quite practicable to replace the original litter by a second and younger litter when necessary, and in this way a rabbit could be kept in full lactation for several months.

Administration of valine. DL-Valine (11·9 mg.) containing 6 μc. of ^{14}C was dissolved in 4 ml. of isotonic saline. The solution was given in four equal doses by intravenous injection (ear vein) at intervals of 30 min. Half an hour after the last injection the litter was removed from the animal and about 6 hr. after the first injection milk and blood (ear vein) samples were taken; the animal was returned to its litter and further samples of milk and blood were withdrawn at suitable intervals.

Administration of lysine. DL-Lysine (543 mg.) containing 11 μc. of ^{14}C was dissolved in 12 ml. of saline and the experiment carried out as described for valine.

Administration of glycine. Instead of dividing the dose, 11·2 mg. of glycine containing 295 μc. of ^{14}C were given as a single injection. In other respects the procedure was as for the other amino-acids except that a milk sample was collected at 6 hr. only and the animal was then killed and the whole of the blood collected.

A further experiment was made with glycine in which 1·8 mg. of glycine containing 5 μc. of ^{14}C were injected into a normal rabbit and blood samples were withdrawn after 4, 6, 8, 10, 16 and 24 hr.

Separation of milk proteins. In the lysine and valine experiments, the milk protein was separated into a casein and a non-casein fraction. The casein was precipitated by addition of a mixture of N-HCl and N-acetic acid (1:2 by vol.) (Van Slyke & Baker, 1918) to the milk previously diluted with 3 vol. of water. The casein separated cleanly at pH 4·6 and was removed by centrifugation. The casein was resuspended in water, centrifuged, and the supernatant added to the non-casein fraction. The casein was then suspended in ethanol, centrifuged, filtered, washed with ether, and air-dried. The pH of the aqueous solution containing the non-casein fraction was adjusted to 7·5. The protein was precipitated by heating to 100°. The precipitated protein was centrifuged, washed twice with water and then with methanol, filtered, washed with ether and dried. The radioactivity of the non-protein fraction was so low that it was not investigated.

In the glycine experiment three fractions were collected instead of two. Casein was separated as previously described, and the pH of the non-casein fraction was brought to 6. An equal volume of saturated $(NH_4)_2SO_4$ solution was added; the precipitate obtained under these conditions is referred to as the 'globulin fraction' (Smith, 1946*b*). After removal of the globulin fraction the solution was saturated with solid $(NH_4)_2SO_4$; the precipitate obtained is referred to as the 'albumin fraction'. In each case the salt-free protein was isolated by heat precipitation. The non-protein fraction which only constituted about 3% of the N of the milk was not investigated.

In order to determine the distribution of protein in rabbits' milk the following method of fractionation was used. After separation of casein as usual, the supernatant was adjusted to pH 7 and the solution saturated with $MgSO_4$. The precipitate obtained is referred to as the 'globulin fraction'. After leaving the precipitate to stand overnight it was separated by filtration through Whatman no. 42 paper and washed with saturated $MgSO_4$ solution. An equal volume of 20% (w/v) trichloroacetic acid was added to the filtrate and the precipitate obtained referred to as the 'albumin fraction'. The filtrate is referred to as the 'non-protein fraction'. The casein was dissolved in dilute alkali and the globulin in water. N determinations (Kjeldahl) were carried out on these two fractions. The amount of albumin was estimated from the difference between the N content of the $MgSO_4$ filtrate and the trichloroacetic acid filtrate. The distribution of protein in a typical sample of rabbits' milk as determined by this method is given in Table 1.

Fractionation of blood proteins. Blood was withdrawn from the ear as required, heparinized and centrifuged. The red cells were washed twice with saline, lysed in water and the protein precipitated by heat after addition of ethanol. When the sample of blood taken was too small to allow proper fractionation of the plasma proteins, the plasma was mixed with the saline washings from the red cells and the protein was precipitated by adjusting the pH to 5·0 and heating.

When the plasma protein was fractionated, the following modification of the procedure described by Howe (1921, 1923) was adopted. The plasma was dialysed against distilled water until free from radioactive non-protein and then freeze-dried. The powder was redissolved in 0·9% (w/v) NaCl, made up to the original volume (21 ml.) and mixed with 10 ml. phosphate buffer (pH 7·8, 0·326M-K_2HPO_4, 0·0243M-KH_2PO_4) and 10 ml. water. Na_2SO_4 (21·6%, w/v) (34 ml.) was added to precipitate the fibrin which was collected on the centrifuge. A further 170 ml. of Na_2SO_4 solution were added to the fibrin-free solution to precipitate the globulin. The precipitate was removed (centrifuge) and the supernatant saturated with solid Na_2SO_4 at 37° to precipitate the albumin. Each protein was heat-precipitated, washed free of salts and dried with ethanol and ether.

For the quantitative estimation of amino-acids in plasma proteins the method of Popják & McCarthy (1946), in which the globulin is precipitated with saturated $MgSO_4$, was used.

Handling of protein samples. Proteins were weighed after drying *in vacuo* over P_2O_5 at room temperature. Unless otherwise stated, samples were hydrolysed in sealed tubes for 18 hr. at 105° in 6N-HCl.

Assay of radioactivity. All samples were plated on 1 or 2 sq.cm. disks and, when accurate comparison was being made between the specific activity of pure samples, counts were prolonged until the standard error of the count was less than 1·5%. All assays were made with disks of infinite thickness. Since the counts with proteins were only approximate, no special drying precautions were taken during counting.

Isolation of lysine. Lysine was isolated as the copper benzylidene complex by the method of Turba (1948). The yields were not as good as those quoted by Turba; e.g. 820 mg. casein gave 55 mg. complex. As the scale was much smaller, this is not surprising. The copper complex was suspended in N-H_2SO_4 and decomposed with H_2S. The solution was boiled and filtered hot, the precipitate being washed with hot water. Sulphate ion was removed from the filtrate as $BaSO_4$ and the aqueous solution of lysine concentrated until 0·5 ml. contained 15 mg. lysine. To this solution was added 1·5 ml. ethanol containing 24 mg. picric acid/ml. Lysine picrate crystallized at once. After recrystallization, this picrate was used for radioactive assay. The same weight (36 mg.) of lysine picrate from each protein was plated on a 1 sq.cm. disk before counting. To eliminate errors of area disks were then exchanged between the two samples and the mean count calculated.

Isolation of valine. After hydrolysis of the protein, the amino-acid mixture was allowed to react with excess of 1-fluoro-2:4-dinitrobenzene (FDNB) as described by Sanger (1945). The mixed dinitrophenyl- (DNP-) amino-acids were extracted into ether and the ether-soluble material fractionated on buffered Celite by the method of Perrone (1951). Preliminary separation of the mixed DNP-amino-acids from 500 mg. protein was satisfactory on a 50 cm. column containing 60 g. Celite 545 (Johns-Manville and Co. Ltd., London, S.W. 1), buffered at pH 6·6 and with ether as solvent. The DNP-valine band was, however, incompletely separated from other products and the material in it would not crystallize. The material was further purified by chromatography on a second column of 45 g. Celite 545 (pH 6·6) using $CHCl_3$ as solvent. The valine band ($R = 0·2$) was collected and the DNP-valine crystallized twice from ether-ligroin, m.p. 129°. From 480 mg. casein we obtained 29·2 mg. DNP-valine and from 520 mg. of a mixed whey protein we isolated 16·2 mg. DNP-valine. Before carrying out the radioactive assay of these two samples they were crystallized to constant and identical melting point, diluted, each with twice its own weight of pure synthetic DNP-L-valine, and again crystallized.

The ease of isolation of DNP-valine is much influenced by the quality of Celite used. With two batches of Celite 545 we had no difficulty, but with a third batch we were unable to get satisfactory fractionation.

Isolation of glycine. For the isolation of glycine from casein, 200 mg. protein were used. Lactalbumin and lactoglobulin contained higher percentages of glycine than casein, and satisfactory results were obtained with 75 mg. protein. The protein was hydrolysed and treated with FDNB as before. Columns of 2·0 or 2·5 cm. diameter were prepared with about 25 g. Celite 545/100 mg. original protein. These columns were buffered with phosphate buffer (975 ml. 0·25M-KH_2PO_4 + 25 ml. 0·25M-Na_2HPO_4), 16 ml. buffer being added to each 25 g. Celite. The Celite was first suspended in peroxide-free ether and stirred mechanically with an efficient stirrer rotating at about 2000 rev./min. The buffer was poured slowly into this suspension and stirring continued for about 15 min. The column was packed as described by Perrone (1951), and the ether then displaced by a $CHCl_3$-*n*-butanol mixture (7 vol. *n*-butanol, 93 vol. $CHCl_3$, saturated with water after mixing). The mixed ether-soluble DNP-amino-acids were dissolved in 10–20 ml. of this $CHCl_3$-butanol-water mixture and transferred to the column. The glycine band ($R = 0·29$) was well separated from all other bands and was collected separately. After removal of solvent *in vacuo* the DNP-glycine crystallized readily, but as the quantity was less than 5 mg., crystallization was considered unsatisfactory as a method of purification. A second column of the same Celite-buffer mixture (2 × 16 cm.) was used for purification of the DNP-glycine. Instead of $CHCl_3$-butanol, washed, water-saturated, peroxide-free ether was used as solvent. The glycine band was collected in a graduated vessel and a suitable volume (about one-tenth of the whole) withdrawn for estimation of glycine. A standard curve for DNP-glycine in 0·5% (w/v) aqueous $NaHCO_3$ (10 ml.) was constructed using a Hilger 'Spekker' photoelectric absorptiometer (filter 601). The curve was linear over the range 200–600 μg. DNP-glycine and all estimations of [^{14}C] glycine were made within this range. For assay of radioactivity, the remaining nine-tenths of the DNP-glycine from the column was diluted with pure synthetic DNP-glycine (about 20 mg./1 mg. [^{14}C]DNP-glycine), the ether was removed *in vacuo*, and the residue crystallized from ether-ligroin. The above method of isolation was found to be more satisfactory than that described by Perrone (1951).

Isolation of serine and threonine. The mixture of DNP-amino-acids which remained on the $CHCl_3$-butanol column after removal of DNP-glycine was washed off with acetone and the acetone solution concentrated *in vacuo*. The residue was dissolved by shaking with a mixture of N-HCl and ether.

After several successive extractions with ether, the combined ether extracts were concentrated, the residue taken up in a few drops of ethyl methyl ketone and diluted with about 10 ml. ether. A Celite 545 column (2·5 cm. diam.) was prepared with 50 g. celite and 30 ml. buffer/100 mg. original protein and with ether as solvent. The buffer (pH 5·9) was a mixture of 2 ml. 0·25 M-Na_2HPO_4 and 3 ml. 0·25 M-NaH_2PO_4. The solution of DNP-amino-acids was transferred to the column and the chromatogram developed, first with ether and then, when a small fast band had been eluted, the ether was displaced by a mixed solvent (150 ml. ethylmethyl ketone diluted to 500 ml. with ether and then saturated with water). After brief development with this solvent the threonine band (R=approx. 0·3) broke away from the main coloured zone. The threonine band was collected and DNP-threonine crystallized from ether-ligroin. On further development of the column a second band (R=approx. 0·15) separated from the main coloured zone. This was identified as DNP-serine by comparison with synthetic DNP-serine and by alkaline hydrolysis (Lowther, 1951) to serine (identified by paper chromatography). By prolonged development of the column with the same solvent mixture, DNP-glutamic acid and DNP-aspartic acid could also be isolated.

The quantity of DNP-serine isolated was too small to permit satisfactory purification by crystallization; purification was effected by chromatography on a second Celite column (10 × 1·5 cm.) using the same buffer and solvent. We satisfied ourselves by experiments with synthetic mixtures that this procedure was justified. The DNP-serine band was collected in a graduated vessel and a suitable volume (about one-twelfth) withdrawn for colorimetric assay (Spekker). A standard curve for DNP-L-serine in 0·5% (w/v) aqueous $NaHCO_3$ (10 ml.) was linear over the range 150–400 μg. and all assays were made within this range. For assay of radioactivity the remainder of the DNP-serine was diluted with a suitable amount of synthetic DNP-L-serine and crystallized from a mixture of ethyl acetate and light petroleum (b.p. 60–80°).

Quantitative estimation of glycine. Glycine was estimated colorimetrically as DNP-glycine after separation as already described. For these quantitative estimations small Celite columns (10 × 0·7 cm.) were convenient, using 3–10 mg. protein. Full details of the method are given in an Addendum to this paper.

Quantitative estimation of valine and lysine. We are indebted to Miss K. de Bouk for these estimations, which were made by the method described in a previous note (de Bouk, 1952).

RESULTS

It is not possible to obtain a continuous supply of milk from a rabbit, and the optimum time for withdrawal of our first milk sample had to be decided. Since we were interested in the possibility that blood protein was the direct precursor of milk protein, the optimum time for first milking would coincide with the maximum in the plasma radioactivity curve. [^{14}C] glycine (5 μc.) was injected into a normal rabbit and blood samples were withdrawn at intervals. The plasma protein was separated and assayed for radioactivity. It is apparent from Fig. 1 that the maximum plasma radioactivity was reached about 6 hr. after injection of [^{14}C] glycine.

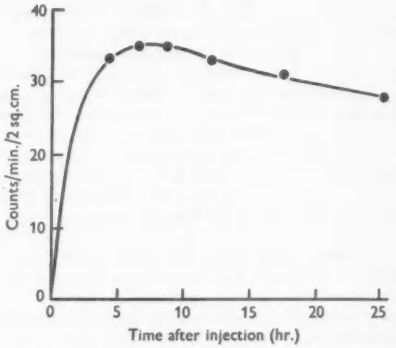


Fig. 1. Radioactivity of plasma protein from a rabbit after administration of [^{14}C] glycine.

A lactating rabbit was injected with 295 μc. [^{14}C] glycine and was milked 6 hr. later. When milking was completed the animal was killed and a small sample of the plasma fractionated as described under Methods. The distribution of radioactivity in blood and milk protein at this time is shown in Table 1.

Table 1. *Radioactivity of blood and milk proteins (counts/min. in 1 sq.cm. sample of infinite thickness) 6–7 hr. after administration of* 295 μc. [^{14}C] *glycine to a* 3·7 *kg. rabbit*

	Milk		Blood‡ activity
	Weight* (%)	Activity†	
Casein	74	1222	—
Albumin	13	1511	313
Globulin	9	1378	776
Fibrin	—	—	221
Haemoglobin	—	—	68

* Fractionated with $MgSO_4$.
† Fractionated with $(NH_4)_2SO_4$.
‡ Fractionated with Na_2SO_4.

Since the picture with glycine is complicated by the rapid metabolic transformation which this amino-acid undergoes, similar experiments were made with [^{14}C] valine and [^{14}C] lysine. The results obtained after injection of valine are shown in Fig. 2. In this case, the animal was not killed and milk samples were collected for 7 days. The results obtained after injection of lysine are given in Fig. 3. In each case the milk proteins reached a much higher activity than the blood proteins.

Where two proteins differ in amino-acid composition it is not possible to make a direct comparison of their content of labelled amino-acid by measuring

the radioactivity of the whole protein since this may be influenced by the amount of the relevant amino-acid in the protein. In order to check on this point, quantitative amino-acid analyses were made on samples of rabbit plasma protein and rabbit milk proteins. The results given in Table 2 show that there are considerable differences in the amino-acid composition of plasma and milk proteins. These differences are, however, quite insufficient to explain the enormous difference in radioactivity between plasma protein and milk protein as shown in Figs. 2 and 3.

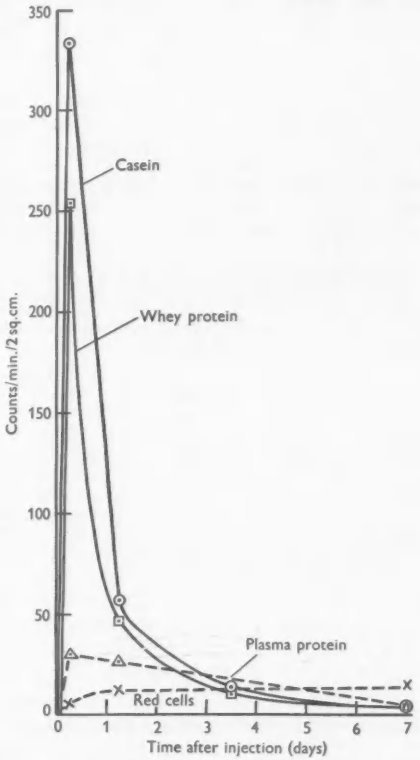


Fig. 2. Radioactivity of milk and blood proteins from a rabbit after administration of [^{14}C] valine.

In another experiment, the radioactive carbon was injected not as free amino-acid but as labelled plasma protein. The rabbit, which had received 295 μc. [^{14}C] glycine, was killed after 7 hr., the plasma was collected, dialysed free of non-protein radioactivity, and freeze-dried. Another lactating rabbit was bled and about 25 % of the blood was replaced by reconstituted radioactive plasma. Samples of blood and milk were then collected at suitable intervals and the radioactivity of the proteins determined. Since there was sufficient radioactive plasma from the one rabbit, two parallel experiments were carried out in which two different lactating rabbits were injected with radioactive plasma. The activities of the proteins isolated in each experiment are given in Table 3. Since the

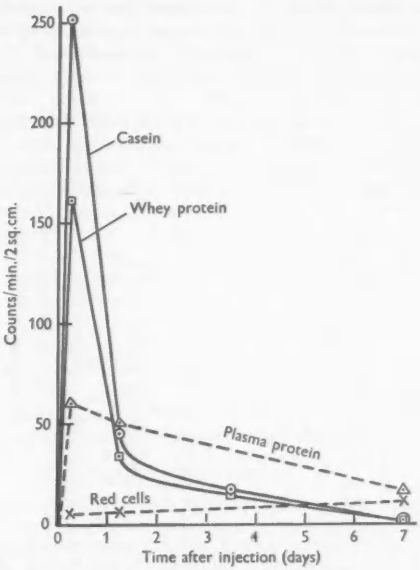


Fig. 3. Radioactivity of milk and blood proteins from a rabbit after administration of [^{14}C] lysine.

Table 2. *Quantitative amino-acid analyses of various rabbit proteins*

(Valine and lysine determined microbiologically, glycine colorimetrically (as DNP-amino-acid). Results expressed as g. amino-acid/100 g. protein.)

	Milk				Plasma*	
	Casein	Whey protein	Albumin*	Globulin*	Albumin	Globulin
Valine	8·0	6·4	—	—	7·8	9·1
Lysine	7·3	5·3	—	—	9·8	6·6
Glycine	1·2	—	4·5	3·7	3·1	2·8

* Fractionated with $MgSO_4$.

Table 3. *Activity of milk and blood protein (counts/min. in 1 sq.cm. sample of infinite thickness) after administration of radioactive ([^{14}C] glycine) plasma protein to rabbits*

	Exp. 1					Exp. 2				
Time after injection ...	1 hr.	2 hr.	8 hr.	31 hr.	1 week	30 min.	1 hr.	6 hr.	30 hr.	1 week
Plasma protein	79	—	53	29	11	67	—	42	23	6·7
Milk, whey protein	—	0·2	1·5	2·1	2·1	—	Inactive	1·0	1·2	1·0
Milk, casein	—	0·3	0·8	1·5	0·7	—	0·9	1·4	1·6	Inactive

counts on the solid samples of the milk proteins were so little above background, a more sensitive method of assay was used for the milk proteins isolated in the second experiment. Mr O'Connor of this Institute kindly determined the radioactivity of the weakly active proteins by conversion of the protein carbon to carbon dioxide and assay in a sensitive gas counter. The results of these experiments are given in Fig. 4.

The results already obtained showed that there were major differences in radioactivity between plasma proteins and milk proteins, but we wished to know also whether there were differences between

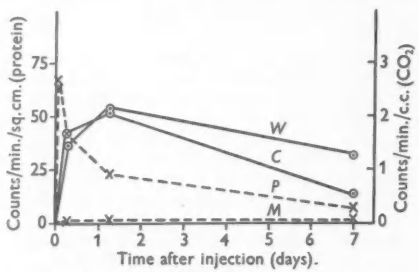


Fig. 4. Radioactivity of milk and plasma protein from a rabbit after administration of radioactive plasma protein ([^{14}C] glycine). Curves *W* and *C*: radioactivity of whey protein and casein as determined in gas counter after combustion. Curves *P* and *M*: radioactivity of plasma and milk protein as determined from infinite-thickness solid samples.

the milk proteins themselves and whether the injected radioactive amino-acid was in complete equilibrium or only in partial equilibrium with the amino-acids of each milk protein. To do this, it was necessary to isolate lysine, valine and glycine from the appropriate protein samples and, after purification, to determine the specific radioactivity of each amino-acid; the results are given in Table 4, the details of isolation and purification are given in the Methods section.

Provided that all the radioactivity of the milk proteins was due to the presence of the injected amino-acid, then it should be possible with the information given in Table 2 concerning the amino-acid composition of the proteins and the radioactivity of the whole protein (Table 1, Figs. 2 and 3) to calculate the specific activity of each of the injected amino-acids. A comparison of the calculated figure with that found by experiment (Table 4)

Table 4. *Specific activity of pure amino-acids (counts/min. in 1 sq.cm. sample of infinite thickness) isolated from hydrolysates of milk proteins obtained 6 hr. after administration of* [^{14}C] *lysine,* [^{14}C] *valine, and* [^{14}C] *glycine*

(The values are calculated with allowance for the mol.wt. of the amino-acid derivative isolated and used for counting and for any inactive carrier used.)

	Glycine	Serine	Valine	Lysine
Casein	28 000	19 900	3050	1700
	29 200	20 100		
Albumin	20 400	13 200	2760*	1525*
	22 600	13 050		
Globulin	25 010	14 750		

* In these cases the whey protein was treated as a single sample as the amount of milk available was considered insufficient to isolate the constituent amino-acids from the albumin and globulin fractions separately.

Table 5. *Calculated and experimental values for the specific activity of amino-acids (counts/min. in 1 sq. cm. sample of infinite thickness) isolated from milk proteins 6 hr. after administration of* [^{14}C] *lysine,* [^{14}C] *valine and* [^{14}C] *glycine*

(The calculated values are $100A/x$, where A = observed activity of whole protein, and x = g. amino-acid residue in 100 g. protein.)

	Casein		Whey protein	
	Found*	Calc.	Found*	Calc.
Lysine	1 700	2 060	1 525	1 800
Valine	3 050	2 600	2 760	2 500
Glycine	28 500	134 000	21 000†	64 000†
			25 000‡	

* Cf. Table 4.
† Value for albumin.
‡ Value for globulin.

would then test the validity of the assumption. The results of such a calculation on the 6 hr. milk protein are given in Table 5. It became obvious that, although the assumption was substantially correct for lysine and valine, it certainly was not correct for glycine. The proteins from the glycine rabbit, therefore, contained some other amino-acid which made a major contribution to the total radioactivity of the protein. Accordingly a casein hydrolysate from the [^{14}C] glycine milk was analysed by single-

dimensional paper chromatography. The strip was sprayed with ninhydrin in the usual way, the position of the various amino-acids marked and the strip scanned for radioactivity by an automatic recording Geiger-Muller counter. The scanning was kindly carried out for us by Mr D. O'Connor. The result (Fig. 5) left no doubt that much of the activity

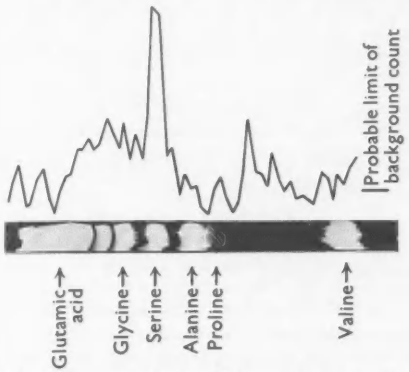


Fig. 5. Radioactivity of chromatogram of hydrolysate of casein obtained from milk of rabbit after administration of [^{14}C] glycine. Hydrolysate chromatographed on paper in 'collidine' solvent, sprayed and coloured with ninhydrin (photograph, below), and drawn under a G.M. counter (curve, above). Owing to the monochrome of the photograph, some of the ninhydrin spots are not distinguishable. Proline is indicated by a lightly hatched area, and one spot is drawn in.

of the protein was due to serine, and accordingly a method was devised for the isolation of this amino-acid from the casein, albumin and globulin residues remaining after isolation of dinitrophenylglycine. The specific activity of the serine is recorded in Table 4.

DISCUSSION

Since the constituents of milk must be transported to the mammary gland in some form or other by the circulating blood, considerable attention has been devoted to arterio-venous changes of amino-acids and proteins in the blood of lactating goats and cows (Folley, 1949; Nikitin, 1949; Kay, 1947; Reineke, Williamson & Turner, 1941; Graham, Peterson, Houchin & Turner, 1937). According to Kay, 'it has been accepted for some time that the total amount of amino-acids taken up as such by the lactating gland is insufficient to account for more than a small fraction of the protein appearing in the milk'. Graham *et al.* (1937) suggested that a globulin fraction of the blood was utilized for milk synthesis and that part of the globulin molecule was used without prior degradation to amino-acids. On the other hand, results reported in the literature suggest that the maximum rate of synthesis of plasma protein is considerably less than would be required to supply all or nearly all the milk protein (Sprinson & Rittenberg, 1949; Robscheit-Robbins, Miller & Whipple, 1945). In interpreting the present results, four possibilities have to be considered: (*a*) milk protein might be derived entirely from plasma protein by minor rearrangement of peptide chains; (*b*) milk protein might be derived entirely from plasma protein via free amino-acids liberated within the cells of the mammary gland; (*c*) milk protein might be derived partly from plasma protein and partly from blood amino-acids; (*d*) milk protein might be derived entirely from free amino-acids without any necessity for the participation of plasma protein.

In attempting to design experiments which might help us to decide between these various possibilities, the use of radioactive isotopes appeared likely to be of great assistance. However, the high cost of radioactive amino-acids prompted us to limit the size of the animal and for this reason the rabbit was chosen. Experiments on lactating rabbits are necessarily a compromise between what is desirable and what is experimentally practicable. For example, it was found to be inadvisable to take milk and blood samples too frequently since too much disturbance of the animal interrupts milk production. The size of the protein samples was also limited so that it was not always practicable to fractionate the milk protein into albumin, globulin and casein. In these cases the milk proteins were separated into two fractions only, a casein fraction and a whey-protein fraction.

The results obtained leave no doubt that of the four possible methods for the biosynthesis of milk proteins two can be excluded. If only minor transpeptidation within pre-existing plasma protein was necessary to convert it to milk protein, then the two proteins would be expected to have approximately the same radioactivity shortly after injection of ^{14}C; the activity/time curve for plasma reaches a maximum after about 6 hr. (Fig. 1) and if plasma protein were the direct precursor of milk protein, the activity/time curve for milk protein would necessarily reach its maximum some time after this and would never rise above the peak of the plasma curve (Hevesy, 1948). Reference to Fig. 2 shows that after injection of [^{14}C] valine the casein and whey-protein activity curves reach values about ten times greater than the plasma curve. The results with lysine (Fig. 3) and glycine (Table 1) confirm the valine experiment and show that plasma protein cannot be the direct and only precursor of milk protein either by minor rearrangement of peptide chains (alternative *a* excluded), or by prior breakdown of the plasma proteins to free amino-acids (alternative *b* excluded).

The results obtained from the injection of labelled

plasma protein also support the exclusion of alternatives (*a*) and (*b*). Thus the maximum labelling of the plasma protein in the two experiments was 79 and 67 counts/min./sq.cm. compared with a maximum activity of the milk proteins of 2·2 and 1·6 counts/min./sq.cm. respectively (Table 3). It was thought that the low activity of the milk proteins might be due to the activity in the plasma being concentrated in a small fraction which was not available for milk-protein synthesis. However, the results given in Table 1 indicate that this was not so. Schoenheimer, Ratner & Rittenberg (1942) obtained similar results after feeding amino-acids labelled with ^{15}N.

While the activity of the casein and whey-protein fractions after injection of the labelled amino-acids was far above that of the plasma it will be observed that in the case of valine and lysine the activity of the whey protein was less, and in the case of glycine more, than that of the casein. The last case is complicated by the fact that much of the glycine is rapidly converted to serine (Results and Table 4). Two explanations of these differences should be considered. First, the amount of the active amino-acid in the two proteins might be different. That this is so is shown in Table 2. Secondly, the two proteins might have been derived from amino-acid precursors of different activity. Before considering this point it is necessary to consider the effect of changes in the total rate of synthesis of milk proteins in the mammary gland on the rate of synthesis of any one of the constituent proteins.

So far as we can tell, all the milk proteins are synthesized in one type of cell (Richardson, 1947; Kay, 1947). There does not appear to be a casein-synthesizing cell, a lactoglobulin-synthesizing cell, etc. However, the possibility must be admitted that for the purposes of protein synthesis different parts of the same cell may have different functions. There is at present no evidence on this point and in our argument it will be assumed that the cell can be considered as a whole. In any brief period (a few hours) the rate of synthesis of each of the milk proteins is likely to bear a constant ratio to the overall rate even if this varies appreciably; thus, if R_1, R_2, R_3, etc., are the rates of synthesis of whole milk protein during successive intervals of time T_1, T_2, T_3, etc., and A_1, A_2, A_3 the rates of synthesis of an individual milk protein A, then

$$\frac{A_1}{R_1}=\frac{A_2}{R_2}=\frac{A_3}{R_3}=k_1.$$

If B_1, B_2, B_3, etc., are the rates of synthesis of a second protein B, then

$$\frac{B_1}{R_1}=\frac{B_2}{R_2}=\frac{B_3}{R_3}=k_2,$$

i.e.
$$\frac{A_1}{B_1}=\frac{A_2}{B_2}=\frac{A_3}{B_3}=\frac{k_1}{k_2}=K. \qquad (1)$$

If at time T_0 we inject any suitable quantity of a radioactive amino-acid, then the concentration of this [^{14}C] amino-acid available for protein synthesis during times T_1, T_2, T_3, etc., can be expressed as S_1, S_2, S_3, etc. The same amino-acid will also be available in the ^{12}C form in variable concentration expressed as M_1, M_2, M_3, etc.

If the rates of synthesis of milk protein A at times T_1, T_2, T_3, etc., are A_1, A_2, A_3, etc., then the specific activity (Sp) of amino-acid S in protein A at time T_n will be

$$Sp=\frac{S_1A_1+S_2A_2+S_3A_3+\ldots S_nA_n}{M_1A_1+M_2A_2+M_3A_3+\ldots M_nA_n}. \qquad (2)$$

The specific activity (Sq) of the same amino-acid in a second milk protein B will be

$$Sq=\frac{S_1B_1+S_2B_2+S_3B_3+\ldots S_nB_n}{M_1B_1+M_2B_2+M_3B_3+\ldots M_nB_n}. \qquad (3)$$

Rewriting Eqn. (1)

$$A_1=B_1K, \quad A_2=B_2K, \quad \ldots, \quad A_n=B_nK.$$

Substituting these values in (2)

$$Sp=\frac{S_1B_1K+S_2B_2K+S_3B_3K\ldots S_nB_nK}{M_1B_1K+M_2B_2K+M_3B_3K\ldots M_nB_nK}. \qquad (4)$$

Eliminating K from (4), $Sp=Sq$ (see (3)).

Thus, even though the overall rate of milk synthesis during the 6 hr. period between injection and milking may vary, the specific activity of the injected amino-acid re-isolated from any two milk proteins should be the same.

We are, of course, neglecting in this argument any breakdown of milk protein, but as the mammary gland is a secretory organ with a high overall output such breakdown can probably be neglected over a period of a few hours. We are also assuming that the amount of amino-acid given by intravenous injection was not sufficient to cause gross changes in metabolism. In the case of valine only 12 mg. of valine was given over a period of 1·5 hr. which would not be expected to be sufficient to cause such changes. In the case of lysine, much more was given (543 mg.) over a similar period, but since substantially the same results were obtained with lysine as with valine it may be assumed that such a large dose did not affect the metabolism significantly. In the case of glycine only a small amount (11 mg.) was injected.

If the theoretical treatment of the specific activities is accepted, we should expect to find after injection of lysine, valine, or glycine, that the specific activity of the relevant amino-acid isolated from casein and from whey protein should be equal. Reference to Table 4 shows that the valine re-isolated from the 6 hr. casein samples had a slightly higher specific activity than the valine from the whey protein. Similarly, the lysine from casein had

a slightly higher specific activity than the lysine from whey protein. In each case the difference is rather small but is probably outside the experimental error; we would hesitate, however, to base any theory on these results alone. After injection of [^{14}C] glycine we re-isolated not only glycine but also serine. Conversion of glycine to serine must take place before protein synthesis so that the net result is equivalent to the injection of two amino-acids simultaneously into the same animal. If we take the casein specific activity figure for each amino-acid as 100 then albumin-glycine = 75, albumin-serine = 65, globulin-glycine = 87, globulin-serine = 73. These differences are well outside the experimental error observed in successive determinations on the same sample (Table 4), and we must, therefore, seek some explanation for them.

We have assumed in our argument that the precursor of milk protein is free amino-acid and that injected radioactive amino-acid will equilibrate with this. However, the specific-activity figures indicate that the amino-acids of milk albumin and globulin were incompletely equilibrated with injected amino-acid, i.e. there must be at least two forms of lysine, valine, glycine or serine used for milk synthesis, one form being, in each case, unable to equilibrate with injected radioactive amino-acid. This 'bound' amino-acid could be represented by either protein or peptide. If we assume that the casein was synthesized entirely from free amino-acid then the amount of bound amino-acid utilized for the synthesis of the whey proteins can be calculated from the differences in the specific activities of the casein and whey-protein amino-acids. If the bound glycine was not radioactive then 25 % of the albumin-glycine and 12 % of the globulin-glycine must have been transferred in this form. However, since the plasma proteins were themselves labelled, the bound glycine would probably have some activity; thus our figures must represent the minimum of each amino-acid supplied in a bound form.

In attempting to decide whether the bound amino-acid is transferred as whole protein or as peptide, the evidence for the transfer of whole protein across membranes must be considered.

It has been shown that young calves absorb into the blood stream immune globulin present in colostrum (Jameson, Alvarez-Tostado & Sortor, 1942; Marrack, 1947; Smith & Holm, 1948), but there is no evidence for the absorption of casein from a similar source. Thus, protein which is homologous, or nearly so, probably can be absorbed from the alimentary tract, but foreign protein such as casein cannot. Dent & Schilling (1947) also came to a similar conclusion from experiments of a different kind in dogs.

If the mammary gland possessed the same type of permeability to proteins as the intestine, then some albumin might be transferred from the serum (Polis, Schmukler & Custer, 1950; Coulson & Stevens, 1950) together with some immune globulin (Smith, 1946*a*, *b*). Together these proteins account for about 5 % of the nitrogen of milk. Thus it is not impossible on present evidence that the bound amino-acid is in the form of protein. However, the results obtained after the injection of labelled plasma protein suggest that the 'bound amino-acid' may be in the form of peptide rather than whole protein. In this experiment the activity of the milk protein was very small compared with that of the plasma protein (Table 3). The low activity of the milk protein could have arisen by a transfer of the [^{14}C] glycine and [^{14}C] serine as protein, peptide, or free amino-acid. If the transfer took place as protein then the activity of the casein would be expected to be much less than that of the whey protein. However, reference to Fig. 4 shows that the activities of the two proteins up to 30 hr. after injection were very similar.

There is at present no direct evidence in support of the theory that the 'bound amino-acid' is transferred as a peptide. However, proteolytic enzymes can catalyse transpeptidation reactions *in vitro* (Bergmann & Fraenkel-Conrat, 1937; Bergmann & Fruton, 1944; Johnston, Mycek & Fruton, 1950*a*, *b*; Fruton, 1950; Hanes, Hird & Isherwood, 1950, 1952; Waley & Watson, 1951; Fruton, Johnston & Fried, 1951), and a transpeptidase is known to exist in the mammary gland of a lactating cow (Morton, 1950). It is therefore quite reasonable to suppose that protein synthesis in the mammary gland involves simultaneous utilization of free amino-acids from the blood stream and peptides derived directly or indirectly from blood protein by such transpeptidation reactions. While this work was in progress, Anfinsen & Steinberg (1951) published some observations on the uptake of $^{14}CO_2$ in the biosynthesis of ovalbumin. Their results also suggest that protein synthesis may take place by way of peptide intermediates.

Emphasis must be placed on the qualitative nature of the present investigation and on its exploratory character. The terms 'albumin' and 'globulin' refer in this work to protein fractions obtained at half and full saturation with ammonium sulphate, and the casein fraction is the mixed protein precipitated at pH 4·7. It is thus possible that within these groups there may be some protein formed entirely from peptides and other formed entirely from amino-acids. We hope to clarify the situation by extension of this type of investigation using a larger animal and by carrying out systematic fractionation of the proteins.

One other point emerges from the present work, the great rapidity of the transformation of glycine into serine (Elliott & Neuberger, 1950). After

injection of [^{14}C] glycine, utilization of this glycine for milk synthesis will commence at once so that the glycine in the milk would be expected to have considerably greater specific activity than serine. We have not yet been able to degrade the DNP-serine obtained from milk to find whether one or two of the carbon atoms are labelled, but if we assume that only one is labelled then the specific activity of this carbon atom can be calculated. Such a calculation made with the figures from Table 4 showed that serine carbon and glycine carbon were of practically the same specific activity. This result certainly suggests that the transformation of glycine into serine is very rapid and that it may be erroneous to talk of a 'glycine pool'.

SUMMARY

1. Valine, lysine and glycine, each labelled with ^{14}C, have been used to investigate the *in vivo* synthesis of milk proteins in the rabbit.

2. It has been shown from the activity/time curves for the milk and plasma proteins that under the conditions of these experiments the blood amino-acids contribute most of the nitrogen for the synthesis of the milk proteins.

3. A comparison of the specific activities of the labelled amino-acids isolated from the radioactive milk proteins indicates that there exist at least two forms of precursors contributing amino-acid residues for the formation of milk protein.

4. Injection of plasma protein labelled with [^{14}C] glycine and [^{14}C] serine resulted in only a very slight activity in the milk proteins.

5. A method for the quantitative estimation of glycine in protein by means of its dinitrophenyl derivative is described in an Addendum to this paper.

The technical assistance of Mr S. Krol throughout the course of this work is acknowledged with pleasure.

REFERENCES

Ågren, G. (1947). *Acta physiol. scand.* **13**, 347.
Anfinsen, C. B. & Steinberg, D. (1951). *J. biol. Chem.* **189**, 739.
Arnstein, H. R. V., Hunter, G. D., Muir, H. & Neuberger, A. (1952). *J. chem. Soc.* p. 1329.
Barton-Wright, E. C. (1951). *J. Inst. Brew.* **57**, 415.
Bergmann, M. & Fraenkel-Conrat, H. (1937). *J. biol. Chem.* **119**, 707.
Bergmann, M. & Fruton, J. S. (1944). *Ann. N.Y. Acad. Sci.* **45**, 409.
Bouk, K. de (1952). *Biochem. J.* **51**, 567.
Bruce, H. M. & Parkes, A. S. (1946). *J. Hyg., Camb.*, **44**, 501.
Bulman, N. & Campbell, D. H. (1951). *Fed. Proc.* **10**, 404.
Callow, R. K. & Work, T. S. (1952). *Biochem. J.* **51**, 558.
Campbell, P. N. & Work, T. S. (1951). *Biochem. J.* **49**, xlvi.
Campbell, P. N. & Work, T. S. (1952). *Biochem. J.* **50**, 449.
Christensen, H. N. (1950). *J. Nutrit.* **42**, 189.
Coulson, E. J. & Stevens, H. (1950). *J. biol. Chem.* **187**, 355.
Danté, W. R. & Thorne, R. S. W. (1949). *J. Inst. Brew.* **55**, 13.
Deasy, C. L., Borsook, H., Haagen-Smit, A. J., Keighley, G. & Lowy, P. H. (1949). *Fed. Proc.* **8**, 194.
Dent, C. E. & Schilling, J. A. (1949). *Biochem. J.* **44**, 318.
Dunn, M. S. & McClure, L. E. (1950). *J. biol. Chem.* **184**, 223.
Elliott, D. F. & Neuberger, A. (1950). *Biochem. J.* **46**, 207.
Fischer, A. (1948). *Biochem. J.* **43**, 491.
Folley, S. J. (1949). *Biol. Rev.* **24**, 316.
Fruton, J. S. (1950). *Yale J. Biol. Med.* **22**, 263.
Fruton, J. S., Johnston, R. B. & Fried, M. (1951). *J. biol. Chem.* **190**, 39.
Graham, W. R., jun., Peterson, V. E., Houchin, O. B. & Turner, C. W. (1937). *J. biol. Chem.* **122**, 275.
Hanes, C. S., Hird, F. J. R. & Isherwood, F. A. (1950). *Nature, Lond.*, **166**, 288.
Hanes, C. S., Hird, F. J. R. & Isherwood, F. A. (1952). *Biochem. J.* **51**, 25.
Harris, J. I. & Work, T. S. (1950). *Biochem. J.* **46**, 582.
Haurowitz, F. (1950). *Chemistry and Biology of Proteins.* New York: Academic Press.
Heidelberger, M., Treffers, H. P., Schoenheimer, R. Ratner, S. & Rittenberg, D. (1942). *J. biol. Chem.* **144**, 555.
Hevesy, G. (1948). *Radioactive Indicators.* New York: Interscience Publ. Inc.
Hotchkiss, R. D. (1944). *Advanc. Enzymol.* **4**, 153.
Howe, P. E. (1921). *J. biol. Chem.* **49**, 93.
Howe, P. E. (1923). *J. biol. Chem.* **57**, 235.
Jameson, E., Alvarez-Tostado, C. & Sortor, H. H. (1942). *Proc. Soc. exp. Biol., N.Y.*, **51**, 163.
Johnston, R. B., Mycek, M. J. & Fruton, J. S. (1950*a*). *J. biol. Chem.* **185**, 629.
Johnston, R. B., Mycek, M. J. & Fruton, J. S. (1950*b*). *J. biol. Chem.* **187**, 205.
Kay, H. D. (1947). *Brit. med. Bull.* **5**, 149.
Klungsøyr, M., Sirny, R. J. & Elvehjem, C. A. (1951). *J. biol. Chem.* **189**, 557.
Krehl, W. A. & Fruton, J. S. (1948). *J. biol. Chem.* **173**, 479.
Lowther, A. G. (1951). *Nature, Lond.*, **167**, 767.
Malin, R. B., Camien, M. N. & Dunn, M. S. (1951). *Arch. Biochem.* **32**, 106.
Marrack, J. R. (1947). *Brit. med. Bull.* **5**, 187.
Morton, R. K. (1950). *Nature, Lond.*, **166**, 1092.
Muir, H. M., Neuberger, A. & Perrone, J. C. (1951). *Biochem. J.* **49**, lv.
Nikitin, V. N. (1949). *Biokhimiya*, **14**, 211.
Nurmikko, V. & Virtanen, A. I. (1951). *Acta chem. scand.* **5**, 97.
Perrone, J. C. (1951). *Nature, Lond.*, **167**, 513.
Polis, B. D., Schmukler, H. W. & Custer, J. H. (1950). *J. biol. Chem.* **187**, 349.
Popják, G. & McCarthy, E. F. (1946). *Biochem. J.* **40**, 789.
Reineke, E. P., Williamson, M. B. & Turner, C. W. (1941). *J. biol. Chem.* **138**, 83.
Richardson, K. C. (1947). *Brit. med. Bull.* **5**, 123.

Rittenberg, D. (1948). *Cold Spr. Harb. Symp. quant. Biol.* **13**, 173.
Rittenberg, D. & Shemin, D. (1946). *Annu. Rev. Biochem.* **15**, 247.
Robscheit-Robbins, F. S., Miller, L. L. & Whipple, G. H. (1945). *J. exp. Med.* **82**, 311.
Sanger, F. (1945). *Biochem. J.* **39**, 507.
Schoenheimer, R., Ratner, S. & Rittenberg, D. (1942). *J. biol. Chem.* **144**, 541.
Silber, R. H. & Porter, C. C. (1949). *J. Nutrit.* **38**, 155.
Simmonds, S. & Fruton, J. S. (1948). *J. biol. Chem.* **174**, 705.
Simmonds, S. & Fruton, J. S. (1949*a*). *Science*, **109**, 561.
Simmonds, S. & Fruton, J. S. (1949*b*). *J. biol. Chem.* **180**, 635.
Smith, E. L. (1946*a*). *J. biol. Chem.* **164**, 345.
Smith, E. L. (1946*b*). *J. biol. Chem.* **165**, 665.
Smith, E. L. & Holm, A. (1948). *J. biol. Chem.* **175**, 349.
Sprince, H. & Woolley, D. W. (1945). *J. Amer. chem. Soc.* **67**, 1734.
Sprinson, D. B. & Rittenberg, D. (1949). *J. biol. Chem.* **180**, 715.
Taylor, S. P., jun., Simmonds, S. & Fruton, J. S. (1950). *J. biol. Chem.* **187**, 613.
Turba, F. (1948). *Hoppe-Seyl. Z.* **283**, 19.
Van Slyke, L. L. & Baker, J. C. (1918). *J. biol. Chem.* **35**, 127.
Virtanen, A. I. & Nurmikko, V. (1951). *Acta chem. scand.* **5**, 681.
Waley, S. G. & Watson, J. (1951). *Nature, Lond.*, **167**, 360.
Work, T. S. (1949). *Proc. roy. Soc.* B, **136**, 159.

ADDENDUM

The Quantitative Estimation of Glycine in Small Samples of Proteins

By S. KROL
National Institute for Medical Research, The Ridgeway, Mill Hill, London, N.W. 7

While the methods developed by Stein & Moore (1948) and Moore & Stein (1949, 1951) for the quantitative determination of amino-acids using starch and cation-exchange resin give excellent results, they are time-consuming and inconvenient if only a single amino-acid is to be estimated.

The present method is a simple adaptation of the method of Sanger (1945) for the detection of 'end groups' of proteins as *N*-2:4-dinitrophenyl-(DNP-) amino-acids. It has been found to be rapid and convenient and to give results which do not vary by more than $\pm 2{\cdot}5\,\%$ in successive determinations. Each analysis is carried out in triplicate on 5–15 mg. protein (according to the glycine content), and several protein analyses can readily be carried through together in 2 working days. The method is both more rapid and more accurate than that described by Callow & Work (1952).

The protein sample is hydrolysed and the resultant amino-acid mixture is allowed to react with excess 1-fluoro-2:4-dinitrobenzene (FDNB). The mixed DNP-amino-acids are dissolved in a suitable organic solvent and the DNP-glycine separated on a buffered Celite column similar to that used by Perrone (1951). The glycine is then estimated colorimetrically as DNP-glycine.

The rate of reaction of FDNB with glycine was studied and the optimum conditions for carrying out the reaction determined. The method has been applied to the estimation of glycine in the milk and plasma proteins of the rabbit.

EXPERIMENTAL

Solvents. B.P. ether was washed once with an equal volume of fresh aqueous solution of $FeSO_4$ (5%, w/v) and six times with equal volumes of water. $CHCl_3$ was washed with six changes of water, distilled and rewashed with one volume of water. *n*-Butanol was freshly distilled and the fraction boiling at $117 \pm 1°$ used. For chromatography, a mixture of $CHCl_3$ and butanol (93:7 by vol.) was saturated with water; this mixture is referred to throughout as CB.

Columns. Celite 545 (Johns-Manville Co. Ltd., London, S.W. 1) (30 g.) was suspended in washed ether and 18 ml. buffer added (0·25 M-KH_2PO_4 97·5 vol. + 0·25 M-Na_2HPO_4 2·5 vol.). The suspension was stirred at high speed for 30 min. A glass tube (1·0 cm. internal diam.) was packed to a height of 15 cm. with the Celite mixture using the tool of Howard & Martin (1950). The ether was allowed to drain until the top of the Celite was almost exposed and the column then washed with CB (10 ml.).

Procedure. The solution, containing 120–500 μg. DNP-glycine, was taken to dryness over P_2O_5 in a vacuum desiccator, dissolved in 1–2 ml. CB and transferred quantitatively to a Celite column. The chromatogram was developed with CB. The glycine band ($R = 0{\cdot}29$) was sharply defined and no effluent was collected until this band was 0·5 cm. from the bottom end of the column. The effluent was now run into a 50 ml. graduated cylinder. The glycine band left the column with the next 5–10 ml. CB, but collection was continued until the volume of CB effluent in the cylinder was 48–49 ml. The volume of CB was adjusted to 50 ml. and exactly 10 ml. 0·5% (w/v) aqueous $NaHCO_3$ added. On shaking the stoppered cylinder, all the colour was transferred to the aqueous layer, which was separated and centrifuged to eliminate any cloudiness caused by organic solvent. The colour intensity of the DNP-glycine in the aqueous phase was measured in a 'Spekker' photoelectric absorptiometer using a violet (601) filter.

Standard curves. In order to determine the loss of DNP-glycine on the Celite column and to check that the loss was constant over the critical range of concentrations, two standard curves were prepared. Samples of a standard solution of DNP-glycine containing 60, 120, 300, 500 and 700 μg. DNP-glycine were analysed by the above procedure, each analysis being made in triplicate. There was a linear

relationship between concentration and colour density over the range 120–500 μg. DNP-glycine (curve *A*, Fig. 1). A second curve was prepared with another set of samples submitted to the complete procedure except for passage through Celite. Over the range 120–500 μg. DNP-glycine the second curve (curve *B*, Fig. 1) was exactly parallel to the first, i.e. the loss was constant over the critical range. Curve *A* was used in all subsequent estimations.

Reaction of FDNB and glycine. Preliminary experiments showed that reaction between FDNB and glycine in aqueous $NaHCO_3$ was slow and a time/reaction curve was accordingly determined. Glycine (38·4 mg.) and L-glutamic acid (192·0 mg.) were dissolved together in water and the volume adjusted to 50 ml. Portions of this solution (0·2 ml.) were mixed with a 10% (w/v) solution of FDNB in methanol (0·2 ml.) and 6 mg. $NaHCO_3$ added. Samples were shaken in 6 × 0·5 in. test tubes for 12 and 30 min., 1, 2 and 4 hr. At the appropriate time, each sample was diluted with water (1 ml.) followed by peroxide-free ether (5 ml.). The solutions were mixed and the ether layer transferred to a clean tube with the aid of a bulb pipette and a rubber teat. The ether was washed with three lots of 1 ml. water, the washings being added to the main aqueous fraction. This fraction was acidified with 3N-HCl (0·5 ml.) and extracted with eight lots of 5 ml. ether. The combined ether extracts were evaporated to dryness *in vacuo* without raising the temperature and the residues dried rapidly in a vacuum desiccator over P_2O_5. Each sample was finally dissolved in 1–2 ml. CB and analysed by the standard procedure. Colour density was plotted against time (Fig. 2). It is apparent from Fig. 2 that reaction was complete in about 4 hr. and this was adopted as the standard time. In order to check that the result was independent of the concentration of glycine, a second series of experiments was made using a smaller quantity of glycine (50 μg.) in a larger volume of reaction mixture (0·5 ml. water, 0·5 ml. of 10% methanolic FDNB, 10 mg. $NaHCO_3$). Reaction was complete (102% recovery) in 4 hr.

Estimation of glycine in proteins. A typical analysis is reported below. Other results are given in the paper of Campbell & Work herewith (Table 2).

Rabbit milk albumin (7·5 mg.) was hydrolysed for 16 hr. at 105° in a sealed evacuated tube with about 0·05 ml. 6N-HCl. The HCl was removed in a vacuum desiccator over KOH and P_2O_5 and the residue diluted to 2 ml. with water. Samples (0·5 ml.) were transferred to 15 ml. test tubes and shaken for 4 hr. with a mixture of FDNB (0·05 g.), methanol (0·5 ml.) and $NaHCO_3$ (about 50 mg.). At the end of this time the mixture was diluted with water (2 ml.) and the extraction procedure described in the previous section followed. The mixed DNP-amino-acids were then transferred in solvent CB to a Celite column. This solvent-buffer system allows ready separation of DNP-glycine. There is a fast yellow band containing the DNP-derivatives of the aromatic amino-acids, lysine, proline, and the higher homologues of glycine; this is followed by a clearly separated glycine band (R=0·29) and a residual band of serine, threonine and the acidic amino-acids remains on the column. The glycine band was collected and estimated as indicated under Procedure. Three successive samples of hydrolysed albumin gave the following results expressed as 'Spekker' drum readings: 0·36, 0·37, 0·36 (equivalent to 4·55 g. glycine/100 g. albumin).

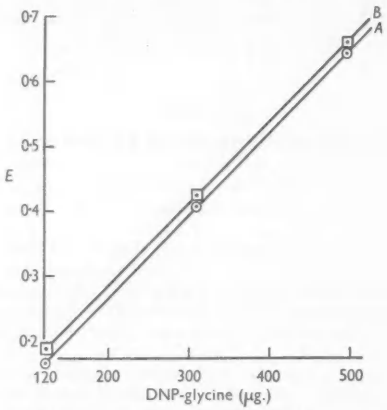


Fig. 1. Relationship between concentration and colour density of DNP-glycine. ⊡—⊡, before passage of DNP-glycine through column. ⊙—⊙, after passage of DNP-glycine through column.

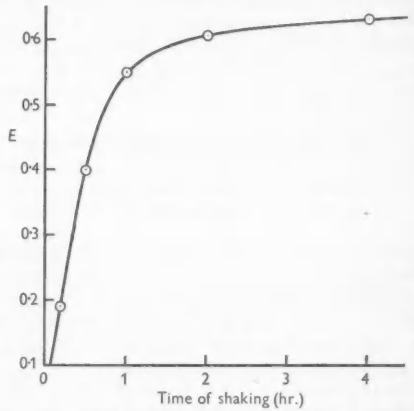


Fig. 2. Relationship between time of reaction and intensity of colour produced in coupling fluorodinitrobenzene and glycine.

REFERENCES

Callow, R. K. & Work, T. S. (1952). *Biochem. J.* **51**, 558.
Howard, G. A. & Martin, A. J. P. (1950). *Biochem. J.* **46**, 532.
Moore, S. & Stein, W. H. (1949). *J. biol. Chem.* **178**, 53.
Moore, S. & Stein, W. H. (1951). *J. biol. Chem.* **192**, 663.
Perrone, J. C. (1951). *Nature, Lond.*, **167**, 513.
Sanger, F. (1945). *Biochem. J.* **39**, 507.
Stein, W. H. & Moore, S. (1948). *J. biol. Chem.* **176**, 337.

Anaerobic and Aerobic Glycolysis in Lactating Mammary Gland and in Nervous Tissue

By C. TERNER
National Institute for Research in Dairying, University of Reading

(*Received* 30 *October* 1951)

The stimulating effect of dinitrophenols on respiration and aerobic glycolysis of tumour slices was described by Dodds & Greville (1933, 1934) and has since been confirmed by many workers (for reviews see Peiss & Field, 1948; Tyler, 1950).

In a study of the effect of 2:4-dinitrophenol (DNP) on the metabolism of lactating mammary gland slices (Terner, 1951*c*), it was observed that the addition of 2×10^{-4} M-DNP caused marked stimulation of respiration when pyruvate was metabolized, but caused inhibition when glucose was the substrate.

In the present paper the results of a study of anaerobic and aerobic glycolysis and of the effect of DNP on the aerobic metabolism of glucose in lactating mammary gland slices and in nervous tissue are reported. It was found that under certain conditions DNP does not influence the breakdown of glucose and the formation of lactic acid in a parallel manner.

Preliminary accounts of part of this work have been given to the Biochemical Society (Terner, 1951*a*, *b*).

EXPERIMENTAL

Material. Mammary gland tissue of lactating rats and rabbits was removed immediately after the death of the animal and placed in ice-cold saline. Slices cut by the method of Stadie & Riggs (1944) were shaken in ice-cold saline to remove as much milk as possible, blotted and portions of approximately equal wet weight (150–200 mg.) were placed in the prepared media in conical Warburg flasks. In some experiments the slices were subjected to a preliminary incubation in saline without added substrates for 1 hr. at 37° in a 100 ml. measuring cylinder aerated by a rapid stream of O_2, and then treated as above. They are referred to as 'depleted' tissue (Terner, 1951*c*). Slices of the grey matter of guinea pig brain were cut by the technique of Deutsch (1936). Ox retina was prepared as described by Terner, Eggleston & Krebs (1950); portions of about 100 mg. wet weight were weighed and placed in the media in Warburg flasks. At the end of the incubation period the flasks were cooled in ice water and 1 ml. of 2 N-HCl was added to the medium (4 ml.). The mammary gland slices and portions of retina were removed, washed rapidly in distilled water, dried in the steam oven and weighed. Portions of the acidified medium were taken for chemical analysis. The brain slices were not removed from the medium; the contents of the flask were poured into a small mortar and ground to a smooth suspension. Portions of this suspension were taken for analysis. The dry weight of the brain slices was taken as 20% of their initial wet weight.

Homogenates. Mammary tissue was homogenized in the homogenizer of Folley & Watson (1948), brain in the homogenizer of Potter & Elvehjem (1936). For dry weight determination portions of the homogenates were evaporated and dried in the steam oven.

Saline media. Phosphate saline without calcium (Krebs & Eggleston, 1940) or bicarbonate saline (Krebs & Henseleit, 1932) were used in tissue slice experiments. A reaction mixture similar to that described by Potter & Le Page (1949) was used for glycolysis in homogenates.

In most aerobic experiments, substrates and inhibitors were placed in the side arm of the Warburg flask and added to the medium after an equilibration period of 10–15 min. In those cases, the incubation periods given in the tables do not include the equilibration period before the addition of the substrates. In anaerobic experiments strictly anaerobic conditions were maintained by a stick of yellow phosphorus in the centre well.

Analytical methods. Pyruvate was estimated by the method of Friedemann & Haugen (1943) after deproteinization with metaphosphoric acid; lactic acid by the method of Barker & Summerson (1941) after deproteinization with trichloroacetic acid; and glucose according to Nelson (1944). Cozymase was estimated spectrophotometrically after reduction with ethanol and alcohol dehydrogenase according to Kornberg (1950). Fluorimetric estimations of the nicotinamide-ribose fraction of cozymase by the method of Huff & Perlzweig (1947) were kindly carried out by Mr D. E. Hughes.

Reagents and preparations. A solution of pyruvic acid was kept as a stock solution and neutralized before each experiment as recommended by Lipschitz, Potter & Elvehjem (1938).

Cozymase preparations used in early experiments were samples of about 30% purity given by Mr D. E. Hughes and by Dr H. McIlwain. Later, a commercial product of 85% purity was obtained from Schwarz Laboratories Inc. Crystalline alcohol dehydrogenase (Racker, 1950) was a gift from Dr E. Racker.

Hexokinase was prepared from baker's yeast according to Meyerhof (1927). Before each experiment 20 mg. of the powder were ground in 2 ml. of distilled water in a glass homogenizer (Potter & Elvehjem, 1936) and the insoluble matter centrifuged off (see Needham, Siminovitch & Rapkine, 1951). The activity of the resulting extract was determined by the manometric method of Colowick & Kalckar (1943); 0·1 ml. of the solution contained 6–8 hexokinase units as defined by Berger, Slein, Colowick, & Cori (1946). The potassium salts of adenosinetriphosphate

Table 1. *Effect of pyruvate and nicotinamide on anaerobic glycolysis of rat mammary tissue*

(Rat mammary gland slices incubated in bicarbonate saline at 37°; gas, 5% CO_2 + 95% N_2; glucose, 0·01 M; pyruvate 0·005 M; nicotinamide, 0·01 M.)

Exp. no.	Tissue portion no.	Additions	$Q^{N_2}_{CO_2}$ (μl./mg. dry wt./hr.) during period (min.) 10–40	40–70	70–100	100–130	10–130
1	1	None	2·7	1·7	1·3	1·2	1·7
	2	Pyruvate	3·4	2·5	1·9	1·8	2·4
	3	Pyruvate, nicotinamide	3·4	2·5	1·8	1·7	2·4
	4	Glucose	4·3	2·8	2·5	2·3	3·0
	5	Glucose, nicotinamide	7·4	5·3	4·2	3·5	5·2
	6	Glucose, pyruvate	6·2	4·8	4·0	3·8	4·7
	7	Glucose, pyruvate	4·9	3·8	3·5	3·2	3·8
	8	Glucose, pyruvate, nicotinamide	8·9	7·5	7·0	6·0	7·3
	9	Glucose, pyruvate, nicotinamide	8·0	7·3	6·0	5·6	6·7

Exp. no.	Tissue portion no.	Additions	$Q^{N_2}_{CO_2}$ (μl./mg. dry wt./hr.) during period (min.) 10–40	40–70	70–100	100–150	10–150
2	1	None	2·9	1·6	1·5	1·1	1·6
	2	Nicotinamide	2·8	2·2	1·8	1·2	1·8
	3	Glucose, nicotinamide	4·8	4·1	2·7	2·4	3·1
	4	Glucose, pyruvate, nicotinamide	8·5	8·2	6·6	5·5	7·3
	5	Glucose, pyruvate, nicotinamide	10·6	10·5	9·1	8·4	9·4

(ATP) and fructose-1:6-diphosphate were prepared from commercial barium salts supplied by Schwarz Laboratories Inc.

Units. All metabolic quotients are expressed in the Q notation (μl./mg. dry wt./hr.). $Q_{\text{lactic acid}}$ denotes the rate of lactic acid production as determined by chemical estimation, while $Q^{N_2}_{CO_2}$ applies to acid formation calculated from changes in manometric pressure.

RESULTS

Anaerobic glycolysis in mammary gland slices. Lactating mammary gland slices of the rat or rabbit, incubated with glucose under anaerobic conditions, produced lactic acid at slow rates ($Q^{N_2}_{CO_2}$ 2–3). In rat tissue the rate of glycolysis was accelerated by the addition of nicotinamide (0·01–0·02 M) and was further increased and stabilized by the addition of pyruvate (0·001–0·005 M). Maximal rates of glycolysis were observed when all factors were present in the medium before incubation started. Delayed addition of glucose, e.g. after 10 min. equilibrium in the bath at 37°, or later, failed to promote rapid glycolysis even when pyruvate and nicotinamide were present from the start.

The instability of anaerobic glycolysis in rat mammary gland slices is also shown in the results of Folley & French (1949), who observed that with glucose as the only substrate, the initially high rate of glycolysis decreased rapidly after short (approx. 30 min.) periods of incubation. In the present experiments, high rates of glycolysis ($Q^{N_2}_{CO_2}$ up to 10) could be maintained for prolonged periods (up to 3 hr.) when glucose, pyruvate and nicotinamide were added (Table 1). Cozymase (10^{-4} to $2{\cdot}5 \times 10^{-4}$ M), in addition to nicotinamide, was no more effective than nicotinamide alone.

In rabbit mammary tissue, however, pyruvate, nicotinamide and cozymase were unable to support a rate of glycolysis ($Q^{N_2}_{CO_2}$) greater than about 3.

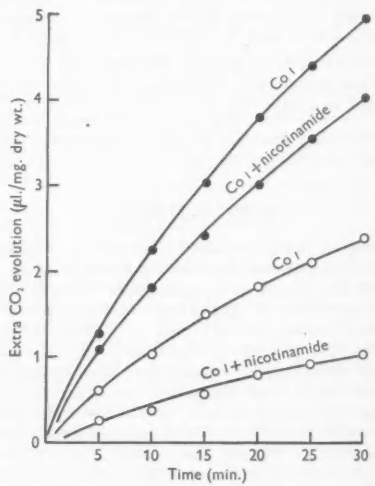


Fig. 1. Effect of nicotinamide on acid production from cozymase. Mammary gland homogenate incubated in bicarbonate saline at 37°; gas, 5% CO_2 + 95% N_2. Cozymase, 1·8 μmoles; nicotinamide, 0·02 M. ●—●, rabbit tissue (10 mg. dry wt.); ○—○, rat tissue (17 mg. dry wt.).

Inactivation of cozymase in mammary tissue. In order to compare the effect of nicotinamide on the breakdown of cozymase in rat and in rabbit mammary tissue, homogenates were incubated in

bicarbonate saline under anaerobic conditions and the extra acid production due to the decomposition of cozymase was measured manometrically as described by McIlwain & Rodnight (1949) (Fig. 1). Comparison of the manometric data with the results of direct estimation of cozymase showed a close relationship between the rates of acid formation and breakdown of cozymase (Table 2).

metrically by the method of Huff & Perlzweig (1947). Analysis of the reaction mixtures by the methods employed by Kornberg & Lindberg (1948) suggests that the pyrophosphatase is also active in the mammary gland of the rabbit (Table 3).

Other factors controlling glycolysis. If the enzymic inactivation of cozymase were the only factor restricting the breakdown of glucose in rabbit

Table 2. *Breakdown of cozymase by mammary tissue*

(Cozymase, 1·95 μmoles incubated with 1·0 ml. of homogenate (1 g. tissue + 7 ml. of water) in bicarbonate saline at 37°; gas, 5% CO_2 + 95% N_2; nicotinamide, 0·04 M.)

Source of tissue	Dry wt./ml. homogenate (mg.)	Flask no.	Inhibitor	Incubation period (min.)	Cozymase broken down (μmoles)	Inhibition (%)	Extra CO_2 evolved (μmoles)	Inhibition (%)
Rat	20·9	1	None	25	1·53	—	1·85	—
		2	Nicotinamide	25	0·62	59	0·76	59
		3	None	50	1·88	—	2·95	—
		4	Nicotinamide	50	0·99	47	1·50	56
Rabbit	17·2	1	None	25	1·53	—	2·45	—
		2	Nicotinamide	25	1·26	18	2·01	18
		3	None	50	1·88	—	3·48	—
		4	Nicotinamide	50	1·88	0	3·20	8

Table 3. *Breakdown of cozymase by rabbit mammary tissue*

(Experimental conditions as in Table 2. Cozymase, 2 μmoles; nicotinamide, 0·02 M. Fluorescence of nicotinamide-ribose fraction of cozymase determined according to Huff & Perlzweig (1947).)

Exp. no.	Flask no.	Incubation period (min.)	Inhibitor	Cozymase recovered (%)	Fluorescence (%)
1	1	0	None	100	100
	2	20	None	48	80
	3	20	Nicotinamide	53	88
	4	40	None	20	58
	5	40	Nicotinamide	26	78
2	1	0	None	100	100
	2	40	None	6	68
	3	40	Nicotinamide	6	82

In rat mammary tissue, the amounts of acid produced were approximately equivalent to the amounts of cozymase broken down, as found by McIlwain & Rodnight (1949) for nervous tissue. In rabbit mammary tissue, however, 1·5–2 moles of acid appeared to be produced for every mole of cozymase broken down. Nicotinamide (0·01–0·04 M) caused 50–80% inhibition of cozymase breakdown in rat tissue, but less than 20% inhibition in rabbit mammary tissue. The results thus suggest that in the rat the inactivation of cozymase is mainly due to a nucleotidase which is inhibited by nicotinamide (Mann & Quastel, 1941; Handler & Klein, 1942), while in the mammary gland of the rabbit cozymase appears to be broken down by another enzyme. Kornberg & Lindberg (1948) have demonstrated the presence in rabbit kidney of an enzyme which hydrolyses the pyrophosphate linkage of cozymase leaving intact the nicotinamide-ribose moiety which can be determined fluori-

mammary slices, it should be possible, by adding large amounts of cozymase, to increase the rate of lactic acid formation during short periods of incubation. This, however, was not found to be the case. Addition of adenosinetriphosphate (0·001 M) was also without effect. Fructose-1:6-diphosphate, however, was broken down to lactic acid more readily than glucose, but, as in the case of glucose, the addition of pyruvate was necessary. Relatively high rates of lactic acid formation were observed in the presence of glucose, fructose-1:6-diphosphate, pyruvate and cozymase (Table 4). Yeast hexokinase appeared to have some effect in accelerating glycolysis when added to the complete system. Its effect was more clearly shown in glycolysing homogenates of mammary tissue in which the rate of lactic acid formation could be increased by hexokinase by more than 100%. Hexokinase not only accelerated the initial rate of glycolysis but, in some experiments, also checked its decrease with time (Table 5).

Table 4. *Anaerobic glycolysis in rabbit mammary tissue*

(Rabbit mammary gland slices incubated in bicarbonate saline at 37°; gas, 5% CO_2+95% N_2. Glucose, 0·005M; pyruvate, 0·005M; fructose-1:6-diphosphate (HDP), 0·005M; cozymase (Coɪ), $1·5 \times 10^{-4}$M; fluoride (0·05M in Exp. 1; 0·025M in Exp. 2); ATP, 0·001M; hexokinase 0·2 ml.; nicotinamide (0·02M) in all cups. Incubation period: 40 min. in Exps. 1 and 2, 60 min. in Exp. 3.)

Exp. no.	Tissue portion no.	Additions	$Q_{\text{lactic acid}}$ (μl./mg. dry wt./hr.)
1	1	Glucose, pyruvate, Coɪ	1·4
	2	Glucose, pyruvate, Coɪ, hexokinase	1·9
	3	Glucose, pyruvate, Coɪ, hexokinase, ATP	2·2
	4	Glucose, pyruvate, Coɪ, hexokinase, ATP, NaF	2·1
	5	Pyruvate, HDP, Coɪ	3·0
	6	Glucose, pyruvate, HDP	3·4
	7	Glucose, HDP, Coɪ	2·6
	8	Glucose, pyruvate, HDP, Coɪ	4·3
	9	Glucose, pyruvate, HDP, Coɪ	4·3
	10	Glucose, pyruvate, HDP, Coɪ, hexokinase	5·4
2	1	Glucose, pyruvate, ATP, NaF	1·3
	2	Glucose, pyruvate, Coɪ, NaF	2·0
	3	Glucose, pyruvate, Coɪ, ATP, NaF	3·0
	4	Glucose, pyruvate, Coɪ, ATP	3·2
	5	HDP, pyruvate, Coɪ	7·0
	6	HDP, pyruvate, Coɪ, NaF	7·3
3	1	Glucose, pyruvate, Coɪ	2·0
	2	Glucose, pyruvate, Coɪ	2·3
	3	Glucose, HDP, Coɪ, hexokinase	1·5
	4	Glucose, HDP, Coɪ, hexokinase	2·0
	5	Glucose, pyruvate, Coɪ, hexokinase	2·1
	6	Glucose, pyruvate, Coɪ, hexokinase	2·6
	7	Glucose, pyruvate, HDP, Coɪ	4·6
	8	Glucose, pyruvate, HDP, Coɪ	5·2
	9	Glucose, pyruvate, HDP, Coɪ, hexokinase	6·5
	10	Glucose, pyruvate, HDP, Coɪ, hexokinase	6·6

Table 5. *Effect of hexokinase on glycolysis in mammary gland and brain homogenates of the lactating rabbit*

(The reaction mixtures (4 ml.) contained: $KHCO_3$, 0·025M: potassium phosphate pH 7·4, 0·0025M; $MgCl_2$, 0·005M; nicotinamide, 0·02M; cozymase (Coɪ), $2·5 \times 10^{-4}$M; ATP, $2·5 \times 10^{-4}$M; glucose, 0·005M; pyruvate, 0·005M; fructose-1:6-diphosphate, 0·001M; 1 ml. homogenate (1 g. tissue + 8 ml. water). Temperature, 37°; gas, 5% CO_2+95% N_2. In Exp. 1 the mammary gland and brain tissue are from the same animal.)

Exp. no.	Tissue	Flask no.	Amount of hexokinase added (ml.)	$Q^{Na}_{CO_2}$ (μl./mg. dry wt./hr.) during period (min.)				
				15–25	25–35	35–50	50–65	15–65
1	Mammary gland	1	None	14·9	8·5	6·0	4·2	8·0
		2	0·05	17·9	10·8	7·6	6·0	9·3
		3	0·1	21·7	15·4	11·6	9·6	13·2
		4	0·2	28·1	23·1	22·0	21·2	22·9
	Brain	5	None	60·0	58·0	36·9	18·5	40·5
		6	0·05	58·5	55·0	28·6	18·2	36·7
		7	0·1	62·0	52·0	24·2	18·1	36·7
		8	0·2	70·6	36·7	26·0	18·7	36·9
		9	0·3	65·6	29·4	24·7	18·1	32·6
				$Q^{Na}_{CO_2}$ (μl./mg. dry wt./hr.) during period (min.)				
				10–20	20–30	30–45	45–60	10–60
2	Mammary gland	1	None	26·7	12·3	9·1	7·0	12·6
		2	0·05	32·0	14·9	12·0	9·0	15·7
		3	0·1	35·1	19·6	14·6	12·2	18·6
		4	0·15	37·7	22·2	18·2	15·9	22·1
		5	0·2	40·0	24·9	20·9	16·9	24·2
		6	0·30	42·1	26·9	21·0	17·3	25·1

The addition of insulin (2·5 mg./100 ml.) and of DNP (10^{-4} M) had no effect on the rate of glycolysis in the absence or presence of hexokinase. On the other hand, in brain homogenates of lactating and non-lactating rats and rabbits, no comparable acceleration of glycolysis was observed on addition of hexokinase; in some brain homogenates inhibition of glycolysis was probably the result of the disturbing effect of large amounts of added hexokinase on the balance of enzymes within the glycolytic system (see Meyerhof & Wilson, 1949).

respiration of mammary gland slices metabolizing glucose, although it greatly increases the respiration in the presence of pyruvate. To investigate this further, the effect of DNP on the metabolism of glucose and pyruvate was studied by chemical analysis of the principal metabolites involved. An experiment in which depleted mammary tissue of the rabbit was used is shown in Table 6. DNP (2×10^{-4} M) almost completely inhibited glucose breakdown and reduced the respiration to the endogenous level (cup 5). The addition of pyruvate

Table 6. *Effect of cozymase and 2:4-dinitrophenol on metabolism of lactating mammary tissue*

(Depleted rabbit mammary gland slices, incubated at 37° in phosphate saline without Ca (Krebs & Eggleston, 1940). Gas, 100% O_2; fumarate (0·002 M) in all cups. Additions: pyruvate 0·005 M; glucose, 0·005 M; nicotinamide, 0·01 M; cozymase (Co I), $2{\cdot}5 \times 10^{-4}$ M; DNP, 2×10^{-4} M. Incubation period: 80 min. $Q_{\text{lactic acid}}$ corrected for lactic acid formed in absence of added substrates.)

Tissue portion no.	Additions	Q_{O_2} (μl./mg. dry wt./hr.)	Q_{pyruvate} (μl./mg. dry wt./hr.)	Q_{glucose} (μl./mg. dry wt./hr.)	$Q_{\text{lactic acid}}$ (μl./mg. dry wt./hr.)
1	None	−2·3	—	—	—
2	Pyruvate	−4·0	−5·8	—	0·3
3	Pyruvate, DNP	−11·8	−6·9	—	0·6
4	Glucose	−5·2	—	−2·0	0·3
5	Glucose, DNP	−2·8	—	−0·3	0·7
6	Glucose, nicotinamide, Co I	−6·2	—	−1·8	0·4
7	Glucose, nicotinamide, Co I, DNP	−3·2	—	−0·1	0·3
8	Glucose, pyruvate	−6·3	−5·8	−1·5	1·0
9	Glucose, pyruvate, nicotinamide, Co I	−6·7	−6·1	−1·3	1·6
10	Glucose, pyruvate, nicotinamide, DNP	−8·8	−4·7	−0·8	1·4
11	Glucose, pyruvate, nicotinamide, Co I, DNP	−10·1	−5·7	−1·4	3·3

Table 7. *Effect of 2:4-dinitrophenol on glycolysis in lactating mammary gland slices*

(Rat mammary gland slices incubated at 37° in bicarbonate saline. Fumarate, 0·02 M, in all aerobic cups. Glucose, 0·005 M; pyruvate, 0·005 M; nicotinamide, 0·02 M; DNP, 5×10^{-5} M. Incubation period: 70 min.)

Tissue portion no.	Additions	Experimental conditions	Q_{glucose} (μl./mg. dry wt./hr.)	Q_{pyruvate} (μl./mg. dry wt./hr.)	$Q_{\text{lactic acid}}$ (μl./mg. dry wt./hr.)
1	None	Aerobic	—	—	0·7
2	Glucose	Aerobic	−4·3	—	0·7
3	Glucose, nicotinamide	Aerobic	−4·2	—	0·6
4	Glucose, DNP	Aerobic	−2·6	—	2·4
5	Glucose, nicotinamide, DNP	Aerobic	−2·8	—	2·8
6	Glucose, pyruvate, nicotinamide	Aerobic	−2·9	−5·8	1·6
7	Glucose, pyruvate, nicotinamide, DNP	Aerobic	−1·6	−6·4	2·4
8	Glucose, nicotinamide	Anaerobic	−3·4	—	6·1
9	Glucose, pyruvate, nicotinamide	Anaerobic	−4·7	−2·2	10·7

The results of chemical determination of lactic acid produced by homogenates agreed closely with the manometric data. In experiments with tissue slices, however, in which the rate of lactic acid production was relatively slow, the additional evolution of gas resulting from the decomposition of added unstable substances and from side reactions introduced large errors which made the manometric measurement of glycolysis impossible and lactic acid was in those cases determined chemically.

Effect of DNP on aerobic glycolysis in mammary gland slices. As previously reported (Terner, 1951*c*), DNP in high concentration (2×10^{-4} M) inhibits the

partly restored the breakdown of glucose and also caused accumulation of lactic acid (cup 10) which was greatly accelerated by the further addition of cozymase (cup 11). High rates of lactic acid formation were thus dependent on the presence of pyruvate, cozymase and DNP, and could not be maintained when one of those factors was omitted.

Although under those conditions lactic acid formation could be greatly increased, the rate of glucose breakdown was not accelerated. The results suggested that aerobic lactic acid formation could, under certain conditions, be increased without a corresponding increase and even with some inhibi-

tion of glucose breakdown. Owing to the relatively low rates of glucose utilization in rabbit tissue, the experiments were repeated under conditions in which larger changes could be observed in the amounts of glucose metabolized. Rat mammary gland slices were incubated in bicarbonate saline without a preceding depletion period (Table 7). A large increase in aerobic lactic acid formation was observed on addition of DNP (5×10^{-5} M) while the breakdown of glucose was inhibited. In fresh rat mammary slices, in contrast to depleted rabbit tissue, the addition of pyruvate, cozymase or nicotinamide was not required under aerobic conditions. DNP had no effect on glycolysis under anaerobic conditions.

Table 8. *Effect of* 2:4-*dinitrophenol on aerobic glycolysis in retina*

(Ox retina incubated in bicarbonate saline at 37°; gas, 5% CO_2+95% O_2. All cups contained glucose (0·005 M) and L-glutamate (0·001 M). Incubation period: 80 min.)

Tissue portion no.	Concn. of DNP (M)	$Q_{glucose}$ (µl./mg. dry wt./hr.)	$Q_{lactic\ acid}$ (µl./mg. dry wt./hr.)
1	0	−11	17
2	2×10^{-5}	−14	22
3	5×10^{-5}	−21	32
4	2×10^{-4}	−26	43

Experiments on other tissues. No inhibitory effect of DNP in the range of concentrations used (2×10^{-5} to 2×10^{-4} M) was observed during a study of aerobic glycolysis in retina, brain or in bull spermatozoa (see Melrose & Terner, 1951). In retina increasing concentrations of DNP accelerated both glucose breakdown and lactic acid formation by approximately equivalent increments (Table 8). In brain tissue the highest rates of glucose breakdown and lactic acid formation were observed when glutamate, nicotinamide, cozymase and DNP were added at the same time. Omission of glutamate, cozymase or of DNP resulted in a relatively low rate of aerobic glycolysis (Tables 9 and 10).

The known effects of glutamate in increasing aerobic lactic acid formation and of pyruvate in reversing the inhibition by glutamate of anaerobic glycolysis in brain slices (Weil-Malherbe, 1938) were also shown in these experiments. The prevention by nicotinamide of the enzymic destruction of cozymase in brain tissue has been shown to increase aerobic glycolysis (McIlwain, 1950). In the present experiments the addition of DNP, glutamate and cozymase could more than double the highest rates of aerobic lactic acid formation observed in the presence of glutamate and cozymase alone. In brain slices in the presence of glutamate, cozymase and DNP, the rate of glucose breakdown ($Q_{glucose}$) under aerobic conditions reached the level observed under anaerobic conditions, i.e. the Pasteur effect was completely inhibited (Table 10).

Table 9. *Effect of cozymase and* 2:4-*dinitrophenol on aerobic lactic acid formation in brain tissue*

(Guinea pig brain cortex slices incubated in bicarbonate saline; gas, 5% CO_2+95% O_2. Complete system: pyruvate, 0·005 M; glucose, 0·005 M; L-glutamate, 0·01 M; nicotinamide, 0·02 M; cozymase (CoI), $1{\cdot}25 \times 10^{-4}$ M; DNP, 10^{-4} M. Incubation period: 80 min.)

Tissue portion no.	Experimental conditions	$Q_{lactic\ acid}$ (µl./mg. dry wt./hr.)
1	Complete system	15·7
2	No DNP	8·9
3	No nicotinamide, no CoI	9·9
4	No glutamate	6·3
5	No glutamate, no DNP	5·5
6	No glutamate, no nicotinamide, no CoI	4·9

Origin of lactic acid formed under the influence of DNP. In view of the inhibitory effect of DNP on aerobic glucose breakdown in mammary tissue the question arises of the mechanism by which the increased amounts of lactic acid are formed.

It is conceivable that the extra lactic acid might be formed by the reduction of pyruvic acid, as a result of the inhibition by DNP of its oxidative removal. This is, however, not the case in mammary tissue, where DNP does not decrease $Q_{pyruvate}$ and does not increase lactic acid formation from pyruvate (see Terner, 1951c). Similarly, in brain

Table 10. *Effect of* 2:4-*dinitrophenol on glycolysis in brain tissue*

(Guinea pig brain cortex slices incubated in bicarbonate saline at 37°. Glucose, 0·005 M; pyruvate, 0·005 M; L-glutamate, 0·005 M; DNP, 2×10^{-4} M; nicotinamide, 0·02 M added with cozymase (CoI), 5×10^{-5} M. Incubation period: 60 min.)

Tissue portion no.	Additions	Experimental conditions	$Q_{glucose}$ (µl./mg. dry wt./hr.)	$Q_{pyruvate}$ (µl./mg. dry wt./hr.)	$Q_{lactic\ acid}$ (µl./mg. dry wt./hr.)
1	Glucose, glutamate	Aerobic	−3·5	—	5·4
2	Glucose, glutamate, DNP	Aerobic	−6·5	—	9·2
3	Glucose, glutamate, CoI, DNP	Aerobic	−8·3	—	12·2
4	Glucose, glutamate, pyruvate, CoI	Aerobic	−3·8	−4·9	5·2
5	Glucose, glutamate, pyruvate, CoI, DNP	Aerobic	−7·2	−4·0	12·5
6	Glucose, pyruvate, CoI	Anaerobic	−7·8	−1·1	16·2
7	Glucose, glutamate, pyruvate, CoI	Anaerobic	−8·4	−3·7	16·0

tissue, under aerobic conditions DNP does not stimulate the reduction of pyruvate to lactate (Table 11). The increased disappearance of pyruvate in the presence of glutamate shown in Table 11 is probably due to transamination and can also be observed under anaerobic conditions (Table 10).

Table 11. *Effect of* 2:4-*dinitrophenol on metabolism of pyruvate in brain tissue*

(Guinea pig brain cortex slices incubated in bicarbonate saline at 37°. Gas, 5% CO_2 + 95% O_2. Pyruvate, 0·005 M; L-glutamate, 0·01 M; DNP, 10^{-4} M. Incubation period: 65 min.)

Tissue portion no.	Additions	$Q_{pyruvate}$ (μl./mg. dry wt./hr.)	$Q_{lactic\ acid}$ (μl./mg. dry wt./hr.)
1	None	—	<0·5
2	Pyruvate	−5·2	2·1
3	Pyruvate, DNP	−5·9	1·6
4	Pyruvate, glutamate	−7·5	2·4
5	Pyruvate, glutamate, DNP	−7·9	2·4

DISCUSSION

Factors controlling glycolysis in mammary tissue

Breakdown of cozymase. Nicotinamide, which in rat mammary tissue inhibits the breakdown of cozymase, is almost ineffective in mammary tissue of the rabbit. Diphosphopyridine nucleotidase, which is specifically inhibited by nicotinamide (Handler & Klein, 1942), therefore appears to be the main cozymase-splitting enzyme in rat mammary tissue. Evidence has been presented suggesting that in the mammary gland of the rabbit the destruction of cozymase is catalysed mainly by the diphosphopyridine pyrophosphatase of Kornberg & Lindberg (1948). This difference in the enzymic composition of the mammary glands of the two species studied may at least partly explain the inability of rabbit mammary tissue to break down glucose, even in the presence of added cozymase, at rates comparable with those attained by rat mammary tissue. In rat mammary gland slices high rates of anaerobic glycolysis can be maintained by nicotinamide without added cozymase, and under aerobic conditions the addition of nicotinamide is unnecessary (Table 7).

The hexokinase reaction. Higher rates of lactic acid formation from hexosediphosphate than from glucose have been observed in cell-free extracts and homogenates (Ochoa, 1941; Utter, Wood & Reiner, 1945). It has been pointed out by Utter *et al.* (1945) that under those conditions 'phosphatase activity may be greater than the hexokinase activity so that little phosphorylation of glucose could occur'. Meyerhof & Wilson (1949) have shown that the rate of glycolysis in tumour homogenates and extracts depends on the balance between the activities of hexokinase and adenosinetriphosphatase. Tissue slices, however, represent a relatively balanced system and anaerobic glycolysis can usually be maintained at high rates with glucose as the only added substrate. The observations that in mammary gland slices hexosediphosphate could give rise to higher rates of lactic acid formation than could glucose, and that added hexokinase could further accelerate glycolysis in slices and in homogenates of mammary tissue, suggest that the rate of glycolysis in this tissue may be limited by a relatively slow hexokinase reaction. Meyerhof (1927) found that the formation of lactic acid from glucose in muscle extracts was facilitated by the addition of yeast hexokinase and attributed this to the lability of muscle hexokinase. Brain hexokinase, on the other hand, was found to be relatively stable (Meyerhof & Wilson, 1949). The present experiments (Table 5) accordingly point to an unstable or deficient hexokinase in mammary tissue. This may be expected, since the lactating mammary gland is under the influence of anterior pituitary and adrenal cortical hormones (see Folley, 1950), which have been shown to inhibit the hexokinase reaction (Cori, 1950). However, added hexokinase did not stimulate glycolysis in brain homogenates of lactating and non-lactating rabbits and rats. No conclusions are drawn from the present results regarding a hormonal inhibition of the hexokinase reaction in mammary tissue, a question which remains a subject for further study.

Effect of pyruvate on glycolysis. Pyruvate has long been known to have a stimulating effect on anaerobic glycolysis (Mendel, Bauch & Strelitz, 1931; Elliott & Henry, 1946). According to Meyerhof & Wilson (1949) pyruvate produces a stimulating effect on lactic acid formation by facilitating the dismutation between pyruvate and triosephosphate in cases in which the formation of pyruvate from glucose is inhibited owing to damage of the enzymes which transform 3-phosphoglyceric acid to pyruvic acid. In the present experiments pyruvate is shown to accelerate and stabilize anaerobic glycolysis in rat and rabbit mammary gland slices (Tables 1 and 3). In depleted mammary gland slices incubated with glucose, pyruvate also increases aerobic lactic acid formation, especially in the presence of DNP and cozymase (Table 6).

Aerobic glycolysis and Pasteur effect

Accumulation of lactic acid under aerobic conditions. The balance sheets presented in this paper show that, as is to be expected, the rate of aerobic lactic acid formation is the resultant of the relative rates of glucose breakdown and pyruvate oxidation. In mammary tissue pyruvate is oxidized as fast as it can be formed from glucose, and therefore lactic acid does not accumulate (Tables 6 and 7). In nervous tissue (Tables 9 and 10) and in spermatozoa

(Melrose & Terner, 1951) in which the glycolysing power of the tissue exceeds its capacity to oxidize the breakdown products of glycolysis, lactic acid is formed by reduction of the excess pyruvate. Thus, the predominance of the glycolytic over the oxidative capacity of a tissue will tend to produce the conditions associated with tissues which have a high aerobic glycolysis, i.e. high rates of glucose breakdown and accumulation of lactic acid. In tissues such as the mammary gland, in which the oxidative capacity is high in relation to their glycolytic power, the rapid aerobic re-oxidation of reduced cozymase at the triosephosphate level will prevent the functioning of the oxidation-reduction step and pyruvate, even if added in excess, will not be reduced to lactic acid (Tables 6 and 7).

The above remarks are based on a scheme which explains the Pasteur effect in terms of a competition between oxygen and pyruvate for the hydrogen of reduced cozymase (Ball, 1939). This scheme, modified by the above considerations relating to the variations in the balance of the glycolytic and oxidative powers of different tissues (see also Warburg, Posener & Negelein, 1924), appears to be in agreement with most experimental observations reported in this paper. It is therefore used as a working hypothesis regarding the mechanism of the Pasteur effect under the present experimental conditions.

Inhibition of the Pasteur effect by DNP. In tissues with a high aerobic glycolysis, e.g. cerebral cortex (in presence of glutamate) and retina, which show a relatively small Pasteur effect for the reasons outlined above, DNP further increases glucose breakdown and lactic acid formation. In mammary tissue, however, DNP in concentrations which produce stimulation of glycolysis and lactic acid formation in other tissues (Tables 6 and 7), causes inhibition of glucose breakdown. Nevertheless, lactic acid formation can be greatly increased by concentrations of DNP inhibitory to glucose breakdown, even in depleted mammary tissue with low glycolytic activity, provided cozymase and pyruvate is added. No explanation is offered for the inhibitory effect of DNP on the breakdown of glucose in mammary tissue in contrast to its accelerating effect in other tissues. However, it would appear that the increased accumulation of lactic acid caused by DNP is not due to its simply accelerating the rate of carbohydrate breakdown.

Since the Pasteur effect is defined as the action of oxygen in diminishing carbohydrate breakdown and in accelerating lactic acid accumulation (see Dixon, 1937) it may be argued that, although DNP inhibits the Pasteur reaction in nervous tissue, it does not do so in mammary tissue in which it does not increase glucose breakdown concomitantly with lactic acid formation. In fact, since under the experimental conditions of Table 7 the rates of glucose breakdown under anaerobic and aerobic conditions in the absence of DNP are of the same order of magnitude (cups 2, 3 and 9), there is, from the point of view of carbohydrate breakdown, no Pasteur effect in mammary tissue. On the other hand, it has generally been considered sufficient, in studies of the Pasteur reaction, to compare the rates of aerobic and anaerobic lactic acid formation (see Burk, 1939). Accordingly, had the present investigations been based on measurements of lactic acid production alone, it might have been inferred that mammary tissue exhibits a considerable Pasteur effect (e.g. Folley & French, 1949), and the increased accumulation of lactic acid under the influence of DNP might have led to the conclusion that the breakdown of glucose was also accelerated, i.e. that the Pasteur effect was inhibited. Various authors have calculated the rates of aerobic carbohydrate breakdown from manometric data of respiration and acid production (see Dixon, 1937; Dickens, 1951). In those calculations the complete oxidation of the substrate was assumed. Although that method may be applicable to many tissues, it would produce erroneous results in others; e.g. in mammary tissue (Terner, 1951*c*), and in spermatozoa (Melrose & Terner, 1951), where ratios of oxygen/substrate were found to be low and to be altered by DNP. The direct determination of the metabolites involved is therefore to be preferred in such studies.

SUMMARY

1. Anaerobic and aerobic glycolysis in mammary tissue has been studied and compared with glycolysis in nervous tissue. The rates of phosphorylation of glucose and of pyruvate formation, and the enzymic inactivation of cozymase were found to limit the rate of anaerobic lactic acid formation in mammary tissue.

2. In the presence of glucose, pyruvate and nicotinamide, high rates of anaerobic glycolysis ($Q_{\text{lactic acid}}$ approx. 10 μl./mg. dry wt./hr.) could be maintained in rat mammary gland slices for periods up to 3 hr.; when pyruvate or nicotinamide were omitted low rates of glycolysis were observed ($Q_{\text{lactic acid}}$ 2–3). Mammary gland slices of the rabbit, in the presence of glucose, pyruvate, nicotinamide and cozymase produced lactic acid at rates $>$ $Q_{\text{lactic acid}}$ approx. 3.

3. The enzymic decomposition of cozymase in homogenates of rat mammary tissue was strongly inhibited by nicotinamide (0·01–0·04 M). In mammary tissue of the rabbit, however, nicotinamide was almost ineffective in preventing the destruction of cozymase. In rabbit mammary tissue, in contrast to rat tissue, the breakdown of cozymase appears to be catalysed by the diphosphopyridine pyrophosphatase shown by Kornberg & Lindberg (1948) to occur in rabbit kidney.

4. In rabbit mammary gland slices, fructose-1:6-diphosphate in the presence of pyruvate and cozymase gave rise to higher rates of anaerobic lactic acid formation than glucose. The addition to this system of glucose and of yeast hexokinase could further increase the rate of glycolysis. In glycolysing homogenates the formation of lactic acid was accelerated by more than 100% on addition of hexokinase. The possibility of hormonal inhibition of the hexokinase reaction in lactating mammary tissue is considered.

5. Under aerobic conditions, 2:4-dinitrophenol (DNP) increased glucose breakdown and lactic acid formation in brain cortex slices and in retina in a parallel manner. In brain slices, in the presence of glucose, glutamate and cozymase, DNP increased the aerobic rate of glucose breakdown to its anaerobic level, thus completely inhibiting the Pasteur effect. In mammary gland slices, however, lactic acid formation could be increased without a corresponding acceleration of glucose breakdown and even with some inhibition. In 'depleted' mammary tissue, DNP caused increased accumulation of lactic acid when glucose, pyruvate and cozymase were present. In the absence of added pyruvate or cozymase, this increase was much smaller or absent.

6. The effect of DNP on the Pasteur reaction is discussed.

I wish to thank Dr S. J. Folley, F.R.S., for his interest in this work and Prof. F. Dickens, F.R.S., for kindly reading the manuscript. My best thanks are due to Mr D. E. Hughes, Dr H. McIlwain and Dr E. Racker for gifts of reagents and to Mr D. E. Hughes for fluorimetric estimations.

REFERENCES

Ball, E. G. (1939). *Johns Hopk. Hosp. Bull.* **65**, 253.
Barker, S. B. & Summerson, W. H. (1941). *J. biol. Chem.* **138**, 535.
Berger, L., Slein, M. W., Colowick, S. P. & Cori, C. F. (1946). *J. gen. Physiol.* **29**, 379.
Burk, D. (1939). *Cold Spr. Harb. Sym. quant. Biol.* **7**, 420.
Colowick, S. P. & Kalckar, H. M. (1943). *J. biol. Chem.* **148**, 117.
Cori, C. F. (1950). *Report of First International Congress of Biochemistry*, p. 19. Cambridge University Press.
Deutsch, W. (1936). *J. Physiol.* **87**, 56*P*.
Dickens, F. (1951). In *The Enzymes*, ed. by Sumner, J. B. & Myrbäck, K. vol. 2, part. 1, p. 675. New York: Academic Press.
Dixon, K. C. (1937). *Biol. Rev.* **12**, 431.
Dodds, E. C. & Greville, G. D. (1933). *Nature, Lond.*, **132**, 966.
Dodds, E. C. & Greville, G. D. (1934). *Lancet*, **1**, 398.
Elliott, K. A. C. & Henry, M. (1946). *J. biol. Chem.* **163**, 361.
Folley, S. J. (1950). In *Modern Trends in Obstetrics and Gynaecology*. London: Butterworth.
Folley, S. J. & French, T. H. (1949). *Biochem. J.* **45**, 117.
Folley, S. J. & Watson, S. C. (1948). *Biochem. J.* **42**, 204.
Friedemann, T. E. & Haugen, G. E. (1943). *J. biol. Chem.* **147**, 415.
Handler, P. & Klein, J. R. (1942). *J. biol. Chem.* **143**, 49.
Huff, J. W. & Perlzweig, W. A. (1947). *J. biol. Chem.* **167**, 157.
Kornberg, A. (1950). *J. biol. Chem.* **182**, 779.
Kornberg, A. & Lindberg, O. (1948). *J. biol. Chem.* **176**, 665.
Krebs, H. A. & Eggleston, L. V. (1940). *Biochem. J.* **34**, 442.
Krebs, H. A. & Henseleit, K. (1932). *Hoppe-Seyl. Z.* **210**, 33.
Lipschitz, M. A., Potter, V. R. & Elvehjem, C. A. (1938). *J. biol. Chem.* **123**, 267.
McIlwain, H. (1950). *Biochem. J.* **46**, 612.
McIlwain, H. & Rodnight, R. (1949). *Biochem. J.* **44**, 470.
Mann, P. J. G. & Quastel, J. H. (1941). *Biochem. J.* **35**, 502.
Melrose, D. R. & Terner, C. (1951). *Biochem. J.* **49**, i.
Mendel, B., Bauch, M. & Strelitz, F. (1931). *Klin. Wschr.* **10**, 118.
Meyerhof, O. (1927). *Biochem. Z.* **183**, 176.
Meyerhof, O. & Wilson, J. R. (1949). *Arch. Biochem.* **21**, 22.
Needham, D. M., Siminovitch, L. & Rapkine, S. M. (1951). *Biochem. J.* **49**, 113.
Nelson, N. (1944). *J. biol. Chem.* **153**, 375.
Ochoa, S. (1941). *J. biol. Chem.* **141**, 245.
Peiss, C. N. & Field, J. (1948). *J. biol. Chem.* **175**, 49.
Potter, V. R. & Elvehjem, C. A. (1936). *J. biol. Chem.* **114**, 495.
Potter, V. R. & Le Page, G. A. (1949). *J. biol. Chem.* **177**, 237.
Racker, E. (1950). *J. biol. Chem.* **184**, 313.
Stadie, W. C. & Riggs, B. C. (1944). *J. biol. Chem.* **16**, 187.
Terner, C. (1951*a*). *Biochem. J.* **49**, ii.
Terner, C. (1951*b*). *Biochem. J.* **49**, lxxiii.
Terner, C. (1951*c*). *Biochem. J.* **50**, 145.
Terner, C., Eggleston, L. V. & Krebs, H. A. (1950). *Biochem. J.* **47**, 139.
Tyler, D. B. (1950). *J. biol. Chem.* **184**, 711.
Utter, M. F., Wood, H. G. & Reiner, J. M. (1945). *J. biol. Chem.* **161**, 197.
Warburg, O., Posener, K. & Negelein, E. (1924). *Biochem. Z.* **152**, 309.
Weil-Malherbe, H. (1938). *Biochem. J.* **32**, 2257.

Gas-liquid Partition Chromatography: The Separation and Micro-estimation of Ammonia and the Methylamines

By A. T. JAMES, A. J. P. MARTIN
National Institute for Medical Research, Mill Hill, London, N.W. 7
and G. HOWARD SMITH*
Low Temperature Research Station, University of Cambridge

(*Received* 15 *November* 1951)

The quantitative micro-analysis of a mixture of ammonia and the methylamines is difficult. A number of colorimetric and other methods exist for the estimation of individual amines, but until recently the only comprehensive scheme (Reay, 1937) depended on the separate analysis of several portions of the mixture after selective precipitation or destruction of one or more of the components—a rather lengthy procedure. Partition chromatography is a promising technique for such a problem and has been used by Fuks & Rappoport (1948) with *n*-butanol-water as the phase pair, on a column of starch mixed with calcium hydroxide. The amines were run as the free bases and were estimated by continuous titration of the effluent from the column. The four amines could be separated from one another but some losses by volatilization occurred during application of the bases to the column. Lagerkvist (1950) separated ammonia and methylamine on a starch column with *n*-propanol-aqueous hydrochloric acid as the solvent system, the positions of the zones being determined by treatment of the eluate with ninhydrin. The recoveries obtained were not stated.

The success which attended the use of gas-liquid partition chromatography in the quantitative micro-analysis of the volatile fatty acids (James & Martin, 1952) suggested its application to the analysis of mixtures of volatile bases. A procedure for the separation of ammonia, methylamine, dimethylamine and trimethylamine is given here; the application of the method to the separation of other aliphatic and of cyclic bases is described in the following paper (James, 1952).

By use of the same general technique as described for the fatty acids, ammonia and the three methylamines may be separated on kieselguhr columns containing a mixture of hendecanol (5-ethylnonan-2-ol) and liquid paraffin as the liquid phase, at a temperature of 78·6°, with only a very slight overlap of the zones of the three methylamines (Fig. 4). The amines emerge from the column in the order of their boiling points (ammonia −33·4°, methylamine −6·5°, trimethylamine 3·5°, dimethylamine 7·4°). It was found that the kieselguhr used as the support for the liquid phase was not inert and adsorbed the amines producing a tail on each zone. Pretreatment of the kieselguhr with methanolic sodium hydroxide prevented most of this adsorption, though enough still occurred to make the zones markedly asymmetric (Fig. 4, curve *B*). No means of overcoming this residual adsorption has yet been found.

The corrected retention volume (V_R^0 as defined by James & Martin, 1952) is a measure of the free energy of association between solute and the liquid phase, both hydrogen bonding and van der Waals forces contributing to this energy of association. The van der Waals forces increase with the number of *N*-methyl groups but each successive group contributes less; the hydrogen-bonding forces vary in a way which in detail is unpredictable. A rough estimate of the van der Waals forces can be obtained from the behaviour of the methylamines on a column having liquid paraffin as the stationary phase, no hydrogen bonding with this solvent being possible (Table 1). The relative hydrogen-bonding power of the methylamines might be expected to run parallel with the order of their basic strengths (dimethyl > methyl > trimethyl > ammonia). Where hydrogen-bonding is possible between solute and solvent (e.g. hendecanol) the order expected from the hydrogen-bonding is different from that expected from van der Waals forces (trimethyl > dimethyl > methyl). The actual order in which the amines will emerge from a column will depend on the relative contributions of the two forces. The factors governing the boiling point sequence are the same as those governing retention-volume sequence in so far as the pure amine constitutes a solvent of properties similar to the liquid phase used on the column. The retention volume of the trimethylamine in a solvent containing hydroxyl groups is greater than would be expected from the boiling point, since hydrogen bonding between the nitrogen atoms in the pure amine is not possible. With hendecanol or hendecanol-15 % (v/v) liquid paraffin as the liquid phase,

* Present address: Biochemical Laboratory, University of Cambridge.

hydrogen bonding is important and the amines emerge in the order of the boiling points. When the hendecanol content is reduced (e.g. to 50% hendecanol-liquid paraffin) the amount of hydrogen bonding is decreased and the van der Waals forces exert a greater effect on zone order. The trimethylamine zone now coincides with the dimethylamine zone (Fig. 5, curve *A*). With a liquid phase of paraffin alone the trimethylamine zone comes after the dimethylamine zone, but the difference is insufficient to allow a useful separation on a 4 ft. column (see Table 1), possibly because of the relatively greater adsorption of dimethylamine on the kieselguhr.

In a more polar solvent such as glycerol, the bonding energy between the glycerol molecules is greater than that existing between the glycerol and amine molecules, the effect being analogous to a high internal pressure forcing out the molecules of higher mol.wt. The order of emergence with a liquid phase of glycerol is thus trimethylamine, dimethylamine, methylamine (Fig. 5, curve *B*). Unfortunately, the effect does not delay the emergence of the ammonia zone enough to prevent it overlapping the dimethylamine zone; presumably this is due to the low basic strength of ammonia. Such a column is therefore useless for resolution of all four bases. A similar effect is observed with a liquid phase consisting of DC 550 silicone-10% hendecanol (Table 1).

Where mixtures of only three of the four amines are encountered, advantage can be taken of particular liquid phases. Table 2 shows those recommended for different amine mixtures. As a test of the method the amines occurring free in *Chenopodium vulvaria* L. (stinking goosefoot) have been investigated and the results of Cromwell (1950) have been confirmed, only ammonia and trimethylamine being found (Fig. 6).

EXPERIMENTAL

Preparation of the columns. Celite 545 was size graded, ignited and acid-washed as described by James & Martin (1952). Before use it was treated with 5% (w/v) methanolic NaOH, decanted and the wet kieselguhr oven-dried at 100°. It was stored in a desiccator over solid NaOH. The liquids used as stationary phases were the following: hendecanol (5-ethylnonan-2-ol, supplied by General Metallurgical and Chemical Ltd., 120 Moorgate, London, E.C. 2), liquid paraffin B.P., DC 550 silicone (Midland Silicones Ltd.) and glycerol. The liquid phase was added to the kieselguhr in the ratio of 3 g. of liquid phase to 7 g. of kieselguhr and the two thoroughly mixed and packed into a 4 ft. 4 mm.-internal-diameter column of the type described by James & Martin (1952). The column efficiencies are of the order of 650 plates, under the conditions of flow rate and temperature shown in Fig. 4.

Burettes. The apparatus used was that described by James & Martin (1952), the titrations being carried out automatically with 0·04 N-H_2SO_4 using 0·007% (w/v) aqueous methyl red solution in the titration cell. Whilst the recording burette is a great convenience, any burette capable of delivering known constant volumes gives perfectly satisfactory results. The titration is then carried out by supplying to the titration cell a certain amount of acid, the time required for its neutralization is noted and a further addition of the same amount of acid is made. (More dilute indicator solution is preferable with this method.) This proves to be easier than reading the burette at constant time intervals. A curve of total acid delivered against time is plotted, the height of each step giving the amount of each component. Three types of suitable burette are illustrated in Figs. 1–3. The first (Fig. 1*a*) consists simply of a small

Table 1. *Relative retention volumes of the methylamines*

(Volumes are relative to that of NH_3, taken as 1.)

Liquid phase (% v/v)	CH_3NH_2	$(CH_3)_3N$	$(CH_3)_2NH$	Temp. (°)
Hendecanol	3·4	4·2	5·2	78·6
Hendecanol-15% liquid paraffin	3·6	4·6	5·8	78·6
Hendecanol-50% liquid paraffin	4·2	7·5	7·5	65
Liquid paraffin-33% hendecanol	3·8	6·8	6·8	65
Liquid paraffin	1·65	2·7	2·7	78·6
Glycerol	2·5	0·47	Approx. 1	100
Silicone DC550-10% hendecanol	1·33	0·36	0·78	65

Table 2. *Conditions recommended for chromatography of amine mixtures*

Amine mixture	Liquid phase	Temp. (°)
Ammonia, mono-, di- and tri-methylamines	Hendecanol-15% (v/v) liquid paraffin	78·6
Ammonia, mono- and di-methylamines Ammonia, mono- and tri-methylamines	Hendecanol-50% liquid paraffin	78·6
Ammonia, di- and tri-methylamines	Hendecanol	78·6
Mono, di- and tri-methylamines	Glycerol or silicone DC550-10% hendecanol	100 65

Erlenmeyer flask, containing the acid, fitted with an inlet and outlet tube. By squeezing the rubber tube fitted to the inlet a drop of acid can be formed on the outlet tube and any number of these drops can be rapidly delivered to the titration cell. The drop size is constant and can be calibrated; the number of drops added to the titration cell to keep the solution near the end point can readily be followed.

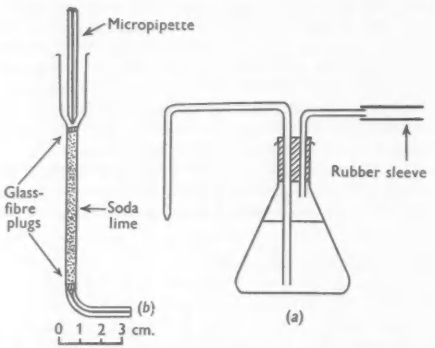


Fig. 1. (*a*) Dropping burette. (*b*) Microcolumn for liberation of the amines from solutions of their salts.

The second (Fig. 2) uses a screw passing through a nut and a cork gland kept in place by sealing wax at the end of the burette. As the screw is rotated by the handle it passes into the burette and the acid is displaced into the titration cell. By counting the number of turns of the screw the total amount of acid delivered is readily ascertained. As glass taps have been found to be unserviceable for long periods because of leakage, the rubber-glass rod valves described by James & Martin (1952) are used. The burette is readily filled by closing the valve leading to the cell and opening the one leading to the acid reservoir, and winding back the screw.

The burette illustrated in Fig. 3 is one which uses the micrometer from an 'Agla' microsyringe (Burroughs Wellcome Ltd.). This type of burette is only suitable when

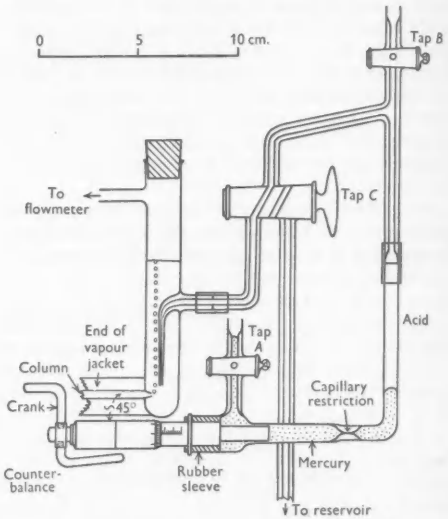


Fig. 3. Micrometer burette and titration cell.

there is a long time interval between the emergence of successive zones. The end of the micrometer barrel is held firmly in the glass tubing by a rubber sleeve. Rotation of the micrometer displaces mercury which in turn forces acid into

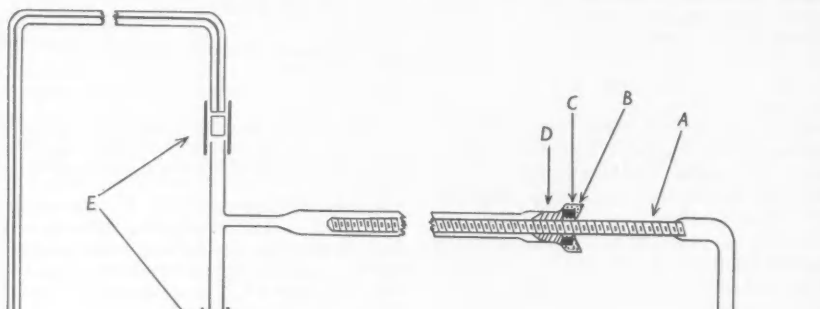


Fig. 2. Screw burette. *A*, screw (2BA thread); *B*, sealing wax; *C*, nut; *D*, cork; *E*, valves.

the titration cell. The taps *A* and *B* are not essential, but enable the initial filling of the burette and removal of air to be performed more easily. The mercury is then adjusted to a suitable level by manipulation of tap *A*. Tap *C* could perhaps be replaced with advantage by the rubber-glass rod valves previously described. The moving scale on the micrometer is divided into hundredths, and can be estimated to one-tenth of a division; with 0·1 N-acid in the burette, a noticeable change in colour of the indicator is observed for a movement of one- or two-hundredths of a rotation. The scale remains in an almost constant position beneath the titration cell, so that the two may be watched simultaneously and readings taken every half min. It is more convenient to operate the burette with the left hand in order to have the right free for recording the results, though the scale marked on the micrometer is then inverted. The burette is filled by reversing tap *C* and winding back the micrometer.

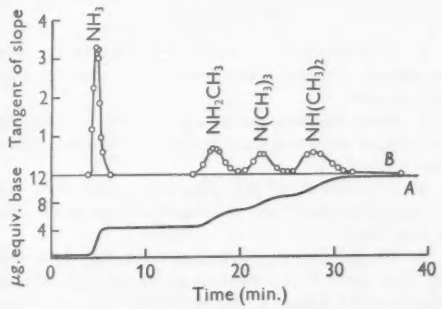


Fig. 4. The separation of ammonia, mono-, tri- and dimethylamines. Curve *A*: experimental curve; curve *B*: curve obtained by graphical differentiation of experimental curve, showing slight tailing of zones. Column length, 4 ft.; liquid phase, hendecanol-15% liquid paraffin (v/v); temp., 78·6°; flow rate of nitrogen, 5 ml./min.; nitrogen pressure, 65 mm. Hg.

Liberation of the free bases from the amine salts. Application of the free amine bases in aqueous solution to the column leads to bad separations and so the following technique has been devised.

A small vertical column terminating in a short horizontal capillary (Fig. 1*b*) is filled with dry soda lime (50 mesh) and a very small plug of glass fibre packed on top of the filling. This extraction column is attached by a piece of pressure tubing to the chromatogram held in the vapour jacket, the dead space at the end of the chromatogram tube being filled by a suitable length of loosely fitting glass rod. Approx. 0·8 N solution (total amines) (0·05 ml.) of the amine salts is run on to the column by placing the micropipette on the glass-fibre plug. When the extraction column has taken up the solution, the plug is washed with two lots of 0·025 ml. of distilled water, the N_2 supply is connected and the gas stream started. The wet part of the column is warmed with a microburner until the visible water front has moved almost to the bottom of the column, the whole tube then being hot. The indicator solution is run into the cell and the titration carried out as usual. The recoveries are approximately theoretical, the reproducibility being ±4%.

Operation of the chromatogram and results. The amines were applied to the chromatograms either as solutions of the free bases in ethanol (3 μl. of a 20% solution) or by means of the technique outlined above, starting from a solution of the amine salts.

The four amines were applied singly and in mixtures to the chromatograms. The retention volume of each zone was measured and corrected to zero pressure difference across the column (V_R^0) by the use of the graphs of the relationship between P_1/P_0 and V_R/V_R^0 given by James & Martin (1952).

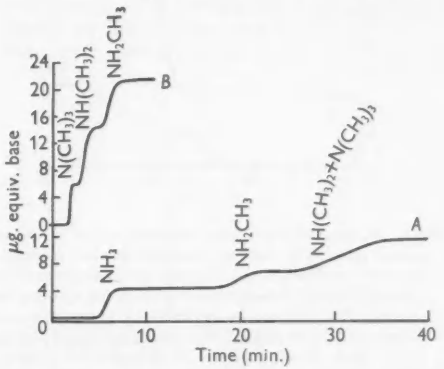


Fig. 5. Curve *A*: the separation of ammonia and the methylamines showing coincidence of trimethylamine and dimethylamine zones on increasing the paraffin content of the hendecanol to 50%. (Column length, 4 ft.; liquid phase, hendecanol-50% liquid paraffin (v/v); temp., 78·6°; flow rate of nitrogen, 3·6 ml./min.; nitrogen pressure, 65 mm. Hg.) Curve *B*: the separation of the methylamines showing the effect of a liquid phase having high internal pressure (glycerol). The amines now appear in order of decreasing molecular weight. (Column length, 4 ft.; liquid phase, glycerol; temp., 100°; flow rate of nitrogen, 4 ml./min.; nitrogen pressure, 60 mm. Hg.)

The corrected retention volumes of NH_3 on each type of column used are shown in Table 3. The solvents used in the vapour jacket to give the temperatures quoted are methanol (65°), ethanol (78·6°) and water (100°). Typical analyses of mixed amines are shown in Figs. 4 and 5.

Table 3. *Corrected retention volumes of* NH_3 *with various liquid phases*

Liquid phase (v/v)	Temp. (°)	V_R^0 (ml.)*
Hendecanol	78·6	37·4
Hendecanol-15% liquid paraffin	78·6	23·2
Liquid paraffin-50% hendecanol	65	24·1
Liquid paraffin-33% hendecanol	65	21·0
Liquid paraffin	78·6	8·2
Glycerol	100	15·0
Silicone DC 550–10% hendecanol	65	210

* Defined by James & Martin (1952).

The burette illustrated in Fig. 3 was used with columns run at room temperature, when of course the time taken was much longer than that shown in Fig. 4. The efficiency of the

column under these circumstances is lower (cf. James & Martin, 1952), but is adequate to separate any three of the four amines provided a suitable liquid phase is chosen (Table 3).

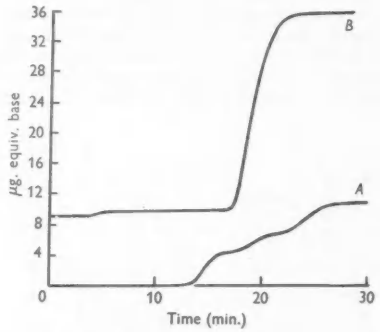


Fig. 6. Analysis of the volatile amines present in *Chenopodium vulvaria* L. Curve *A*: standard mixture of mono-, di- and tri-methylamines. Curve *B*: chromatogram of the amines present in an acid extract of the plant, showing the presence of only ammonia and trimethylamine. (Column length, 4 ft.; liquid phase, hendecanol-15 % liquid paraffin (v/v); temp., 78·5°; flow rate of nitrogen, 5·7 ml./min.; nitrogen pressure, 80 mm. Hg.)

The lower limit of quantity of amine that can be detected with this technique is 0·3 μg. equiv., i.e. 2 μg. of NH_3, 4 μg. of monomethylamine, 7 μg. of dimethylamine and 8 μg. of trimethylamine. The maximum amounts of amines that still allow good separations are 160 μg. of NH_3, 180 μg. of monomethylamine, 180 μg. of dimethylamine and 220 μg. of trimethylamine, using a 4 ft. 4 mm. internal-diameter column.

The estimation of the amines present in Chenopodium vulvaria *L.* The leaves from four plants of *C. vulvaria* L. (approx. 100 g.) were minced in a Waring Blendor and extracted with 0·001 N-HCl (1 l.). The extract was filtered, centrifuged and then made alkaline with NaOH. Air was drawn through the mixture into a wash bottle containing 0·001 N-HCl. At the end of an hour the wash bottle was removed and its contents evaporated almost to dryness. The solution was made up to 2 ml., and 0·1 ml. portions were taken for analysis. The results are shown in Fig. 6.

SUMMARY

1. A technique for the micro-estimation and separation of ammonia and the three methylamines is described.

2. Some factors influencing the relative positions of amines on gas-liquid partition chromatograms are discussed.

3. A method of applying the amines to the columns starting from a solution of the amine salts is described.

4. All the curves illustrated have been obtained by the use of the automatic recording burette described by James & Martin (1952). Three types of manual microburette are now described, together with appropriate titration procedures.

Our thanks are due to Dr B. T. Cromwell for the gift of the stinking goosefoot. Part of this work (by G.H.S.) was carried out as part of the programme of the Food Investigation Organization of the Department of Scientific and Industrial Research.

REFERENCES

Cromwell, B. T. (1950). *Biochem. J.* **46**, 578.
Fuks, N. A. & Rappoport, M. A. (1948). *C.R. Acad. Sci. U.R.S.S.* **60**, no. 7, 1219.
James, A. T. (1952). *Biochem. J.* **52**, 242.
James, A. T. & Martin, A. J. P. (1952). *Biochem. J.* **50**, 679.
Lagerkvist, U. (1950). *Acta chem. scand.* **4**, 543.
Reay, G. A. (1937). *Rep. Food Invest. Bd., Lond.*, p. 69.

Gas-liquid Partition Chromatography: the Separation of Volatile Aliphatic Amines and of the Homologues of Pyridine

By A. T. JAMES
National Institute for Medical Research, Mill Hill, London, N.W. 7

(*Received* 1 *January* 1952)

Previous papers (James & Martin, 1952; James, Martin & Howard Smith, 1952) have described the separation of volatile fatty acids and also of ammonia and the methylamines by means of gas-liquid partition chromatography. This study has now been extended to include higher aliphatic amines and pyridine homologues.

Aliphatic amines

By the use of columns having liquid paraffin as the stationary phase a great many primary, secondary and tertiary aliphatic amines may readily be separated from one another in amounts between 0·3 and 10 μg. equiv. In Fig. 1, curve *A* shows the

separation of methylamine, ethylamine, *iso*propylamine, *n*-propylamine, *iso*butylamine, *n*-butylamine, di*iso*propylamine, *iso*amylamine and *n*-amylamine at a temperature of 100°. The first few amines are not completely resolved, as the column has been run deliberately at a flow rate higher than that suitable for such separations in order to show as many amines as possible on a reasonable time scale. Diethylamine, *sec.*-butylamine and *iso*butylamine have retention volumes (James & Martin, 1952) so similar that they cannot be resolved on such a 4 ft. column. Curve *B* (Fig. 1) shows the separation of

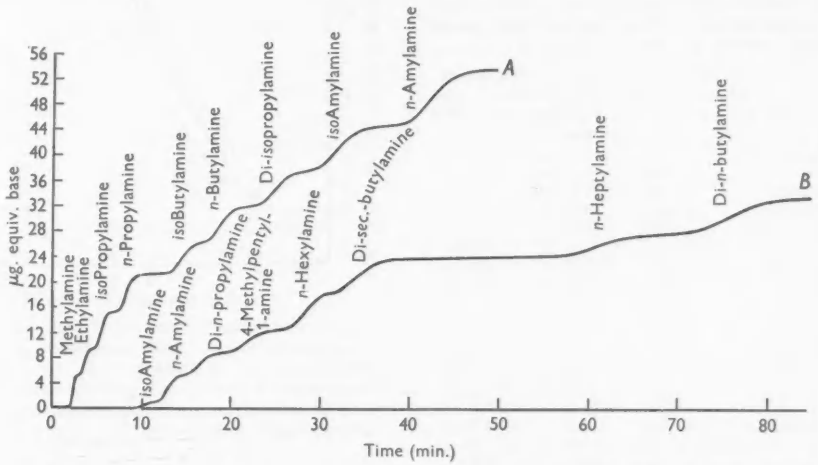


Fig. 1. The separation of aliphatic amines at a temperature of 100°, using a 4 ft. column with liquid paraffin as the stationary phase. Curve *A*: nitrogen pressure, 75 mm. Hg; nitrogen flow rate, 5·7 ml./min. Curve *B*: nitrogen pressure, 225 mm. Hg; nitrogen flow rate, 18·7 ml./min. For data on behaviour of other amines see text and Table 1.

*iso*amylamine, *n*-amylamine, di-*n*-propylamine, 4-methylpentyl-1-amine (*iso*-hexylamine), *n*-hexylamine, di-*sec.*-butylamine, *n*-heptylamine and di-*n*-butylamine at 100° and at a higher flow rate than that used to obtain curve A. In this case triethylamine and *iso*amylamine run together as do di-*sec.*-butylamine, di*iso*butylamine and *cyclo*hexylamine. Tri-*n*-propylamine overlaps *n*-heptylamine. Table 1 gives a list of the retention volumes of the amines relative to that of ethylamine together with their boiling points.

The regularity of behaviour of the aliphatic amines on such paraffin columns is demonstrated by Fig. 2, where the logarithm of the relative retention volume (retention volume of amine/retention volume of ethylamine) is plotted against the number of carbon atoms for a series of primary, secondary and tertiary amines.

The retention volumes (V_R) of the primary straight-chain homologues above ethylamine increase by a factor of 2·1–2·2 for an increase in chain length of one —CH_2 group (a factor of 1·9–2·0 was found for the volatile fatty acids by James & Martin, 1952). The primary *iso*alkylamines show the same factor, as would be expected. The constancy of this factor shows that the van der Waals forces involved in interaction between the carbon chain of the amines and paraffin liquid phase on the columns increase by a constant amount for each additional —CH_2 group. The fact that the primary *iso*alkylamines are separated by a constant factor (1·3–1·4) from their straight-chain isomers indicates the difference in van der Waals interactions between the groups —$CH_2.CH_2.CH_3$ and —$CH(CH_3)_2$, and the carbon chains of the paraffin. The poor separation of *sec.*-butylamine and *iso*butylamine (Table 1) and of di-*sec.*-butylamine and di*iso*butylamine indicates that there is a much smaller difference of energy of interaction with the liquid phase between the groups —$CH_2.CH(CH_3)_2$ and —$CH(CH_3).CH_2.CH_3$. The same effect is shown with the fatty acids *iso*valeric acid and α-methylbutyric acid (James & Martin, 1952). The difference, however, should be sufficient to allow the two amines to be separated in an 11 ft. column (James & Martin, 1952).

The slope of the line connecting the points for the secondary straight-chain amines is slightly less than that connecting the points for the primary amines (Fig. 2). The slope of the tertiary amine line is markedly less, confirming the suggestion made by James *et al.* (1952) that in series NH_2R, NHR_2, NR_3 each additional alykl group contributes less to the

van der Waals forces of interaction with the liquid phase of the column.

A non-polar liquid phase such as paraffin allows no hydrogen bonding between molecules of solute and solvent and so the separations are due solely to differences in the van der Waals forces between the solute and solvent. (This ignores any hydrogen bonding between the solute and the kieselguhr support for the liquid phase, which is, in fact, small.)

Table 1. *Retention volumes of amines relative to that of ethylamine with non-polar and polar liquid phases in the columns at* 100°

(Those amines marked with the same small letters cannot be completely resolved on a 4 ft. column.)

Amine	B.p. (°)	Column liquid phase: Paraffin		Lubrol MO	
Methylamine	−6·5	0·61		0·68	*b*
Dimethylamine	7·4	0·93	*a*	0·81	
Ethylamine	16·6	1	*a*	1	
Trimethylamine	3·5	1·17		0·67	*b*
*iso*Propylamine	34	1·49		1·42	
n-Propylamine	48·7	2·20		2·10	*c*
Diethylamine	55·5	3·25	*d*	2·10	*c*
sec.-Butylamine	63	3·57	*d*	2·8	*e*
*iso*Butylamine	68	3·70	*d*	3·1	*e*
Ethylenediamine	118	4·65	*f*	15·5	
n-Butylamine	77·8	4·7	*f*	4·4	
Ethanolamine	172·2	5·25		25·8	
Di*iso*propylamine	83	6·8		3·00	*e*
Triethylamine	89·5	8·6	*g*	3·1	*e*
*iso*Amylamine	95	8·8	*g*	7·3	*h*
n-Amylamine	104	10·5		9·7	
Di-*n*-propylamine	110·7	13·2		7·2	*h*
4-Methylpentyl-1-amine	123·9	17·8		13·8	
n-Hexylamine	132·7	22·6		19·6	
Di-*sec.*-butylamine	132·0	27·6	*i*	9·74	*j*
*cyclo*Hexylamine	134	28·4	*i*	25·9	
Di*iso*butylamine	139	30·4	*i*	10·9	*j*
n-Heptylamine	158·3	49·5	*k*	40·05	
Tri-*n*-propylamine	156	49·6	*k*	15·1	
Di-*n*-butylamine	159	62·1		27·6	
Tri-*n*-butylamine	214	—		85·5	
Benzylamine	185	81·0		91·5	

Primary and secondary amines contain both active hydrogen atoms (---H—N=) and donor atoms (---N≡) capable of forming hydrogen bonds with suitable groups, e.g. the hydroxyl group of an alcohol, the resulting association complexes being of the types (*a*) R_2N—H---O(H)—R′ and (*b*) R_2N---H—O—R. With solvents such as ethers, which unlike alcohols contain only donor atoms, only one type of hydrogen bonding is possible, namely, (*c*) R_2N—H---O—R′(R″). Tertiary amines possess no active hydrogen atoms and thus form associated complexes only with alcohols (type (*b*)) and not with ethers. 'Lubrol MO' (a polyethylene-oxide-long-chain alcohol condensate) probably has the repeating structure

$$—[O—CH_2CH_2]_n—O—CH_2CH_2—O—R'$$

and with few if any hydroxyl groups. Only primary and secondary amines would be expected to show strong hydrogen bonding with this solvent. On this basis it would be expected that primary and

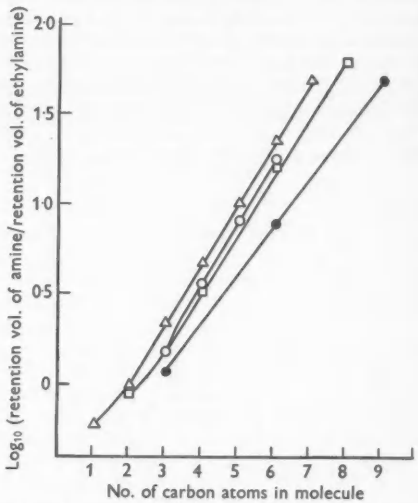


Fig. 2. The relationship between $\log_{10}$ (retention volume of amine/retention volume of ethylamine) and total number of carbon atoms in the amine molecule. △—△, primary straight-chain amines; ○—○, primary *iso*alkylamines; □—□, secondary straight-chain amines; ●—●, tertiary straight-chain amines.

secondary amines would have greater retention volumes on columns having Lubrol MO as the stationary phase than on columns having liquid paraffin as stationary phase, whilst tertiary amines would have similar retention volumes on both

Table 2. *The effect of hydrogen bonding on the corrected retention volumes of primary, secondary and tertiary amines with two different liquid phases on the columns at* 100°

Amine	V_R^0 (ml.)*: Paraffin	Lubrol MO
n-Butylamine	90	234
Di-*n*-propylamine	250	383
Trimethylamine	22	35
Tri-*n*-propylamine	960	800

* Defined by James & Martin, 1952.

columns (assuming the van der Waals forces to be of the same order in both solvents). Table 2 gives a list of the corrected retention volumes of four representative amines on Lubrol MO and liquid-paraffin columns, showing the expected relationship. The exceptional behaviour of trimethylamine is explicable if it is assumed that the H atoms of the

methyl groups are 'active' enough to allow hydrogen bonding of the type $(CH_3)_2NCH_2$—H···O—R′(R″). This suggestion is supported by a comparison of the boiling points of trimethylamine (3·5°) and trimethylmethane (2-methylpropane) (−10·2°) which suggests that hydrogen bonding of the trimethylamine molecules may occur. The boiling points of triethylamine (89·5°) and triethylmethane (3-ethylpentane) (93·3°) are much closer so that it can be assumed that no hydrogen bonding occurs with amines higher than trimethylamine.

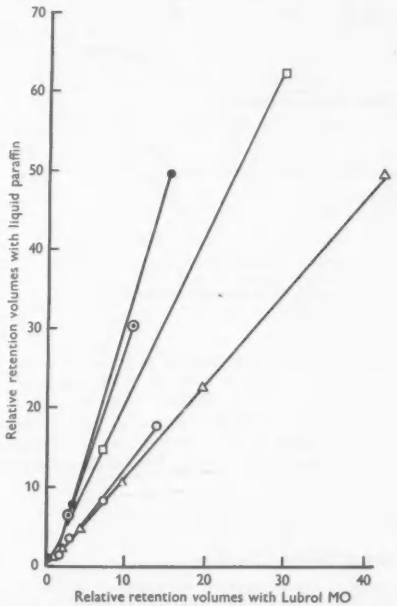


Fig. 3. The relationship between relative retention volumes (retention vol. of amine/retention vol. of ethylamine) for aliphatic amines on columns having stationary liquid phases of (*a*) liquid paraffin and (*b*) Lubrol MO, showing the relative speeding up of secondary and tertiary amines on changing from a solvent not allowing, to one allowing, hydrogen bonding. △—△, primary straight-chain amines; ○—○, primary *iso*alkylamines; □—□, secondary straight-chain amines; ⊙—⊙, secondary *iso*-alkylamines; ●—●, tertiary straight-chain amines.

The order of hydrogen-bonding strength probably follows that of basicity of the amines (primary and secondary > tertiary) and, on columns having Lubrol MO as the stationary phase, secondary and tertiary bases run more rapidly with respect to a given primary amine than on columns having liquid paraffin as the stationary phase. In Table 1 is shown a comparison of relative retention volumes of a series of 27 amines on both types of column. Fig. 3 shows the relationship between relative retention volumes on the two types of column for a series of primary, secondary and tertiary amines. The values for the three types of amine lie on straight lines showing the expected relationship, namely, slope of the line for tertiary amines greater than that for secondary amines greater than that for primary amines. The contribution to the solvation energy of the amines made by hydrogen bonding is still greater in the case of a diacidic amine such as ethylenediamine so that it runs much more slowly on Lubrol MO (relative $V_R = 15·5$) than on paraffin (relative $V_R = 4·65$). In this case the solvent paraffin is so unlike the pure solute ethylenediamine that the amine (mol.wt. 78) behaves similarly to *n*-butylamine (relative $V_R = 4·7$, b.p. 77·8°, mol.wt. 83). Lubrol MO has solvent properties much more akin to pure ethylenediamine so that the base (b.p. 118°) behaves similarly to 4-methylpentyl-1-amine (relative $V_R = 13·8$, b.p. 123·9°, mol.wt. 101).

Identification of aliphatic amines

This property of hydrogen bonding with the solvent can be used to decide whether a given amine is primary, secondary or tertiary, or diacidic by measuring its relative retention volume on the two types of column and finding on which line of the graph in Fig. 3 the values fall. In addition, where zones of two or more amines of differing basic strength overlap on liquid paraffin columns, the amines can be separated by re-running on Lubrol MO columns. In this way dimethylamine and ethylamine; diethylamine and *iso*- or *sec*.-butylamine; ethylenediamine and *n*-butylamine; triethylamine and *iso*amylamine; *cyclo*hexylamine and di*iso*- or di-*sec*.-butylamine, can be separated. Of the 27 amines examined only two pairs, *iso*- and *sec*.-butylamine; di*iso*- and di-*sec*.-butylamine cannot be separated on 4 ft. columns of either solvent.

Table 3. *Factors of increase in relative retention volume for an increase of chain length of one* —CH_2 *group*

	Stationary liquid phase	
	Paraffin	Lubrol MO
Primary amines	2·2	2·1
Secondary amines	2·1	1·9–2·0
Tertiary amines	1·80	1·71

In Table 3 the increases in retention volume for an increase of chain length of one —CH_2 group in primary, secondary and tertiary amines are compared for both types of column. The constancy of these factors allows the prediction of position of any primary, secondary or tertiary amine (having straight or branched chains) provided the retention volume of any other amine in the correct homologous series is known.

Pyridine homologues

Fig. 4 (curve *A*) shows the separation of pyridine, α-picoline, γ-picoline, 2:6-lutidine, 2:4-lutidine and 2:4:6-collidine on a 4 ft. column at 137° using liquid paraffin as the stationary phase. β-Picoline and γ-picoline cannot be separated; the two zones overlap completely. In Table 4 a comparison of the silicone DC 550, Lubrol MO and liquid paraffin as stationary phases is made and it can be seen that liquid paraffin is superior, in that it allows a separation of γ-picoline and 2:6-lutidine. The hydrogen-bonding powers of pyridine, α-picoline, β-picoline and γ-picoline are very similar, so that transferring from a solvent with little to one with high hydrogen-bonding power has little or no effect on their relative position. 2:6-Lutidine, 2:4-lutidine and 2:4:6-collidine probably have a decreased hydrogen-bonding power so that they run more rapidly relative to pyridine on columns allowing hydrogen bonding.

The relative retention volumes of these pyridine homologues may be easily related to those of the aliphatic amines as pyridine has a retention volume relative to ethylamine of 10·5 (at 100°) on paraffin columns, and 10·3 on Lubrol MO columns.

The curves *B*, *C* and *D* (Fig. 4) show the behaviour of commercial samples of 2:6-lutidine, 2:4-lutidine and 2:4:6-collidine. The 2:6-lutidine fraction contains α-picoline, γ- and/or β-picoline, and 2:6-lutidine; the 2:4-lutidine fraction contains a small amount (approx. 4 %) of γ- and/or β-picoline and the 2:4:6-collidine contains a small amount of 2:4-lutidine. The increased width of the collidine zone shows more than one of its isomers to be present. This method of analysis of coal-tar fractions should allow some approach to be made to the standardization of the lutidine and collidine fractions used in paper chromatography, where considerable variations in R_F values are found when different samples are used.

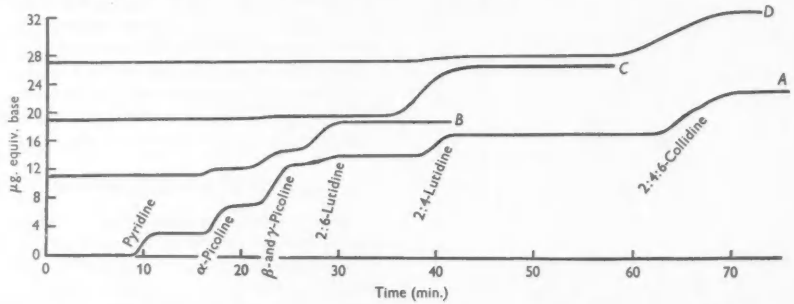


Fig. 4. The separation of pyridine homologues on a 4 ft. column at 137° having liquid paraffin as the stationary phase. (Nitrogen pressure, 19 cm. Hg; nitrogen flow rate, 15 ml./min.) Curve *A*: bases in order of emergence from the column: pyridine, α-picoline, β- and γ-picoline, 2:6-, 2:4- and 2:5-lutidine, 2:4:6-collidine. Curve *B*: analysis of a commercial sample of 2:6-lutidine showing the presence of α-picoline, γ- and/or β-picoline and 2:6-lutidine. Curve *C*: analysis of a commercial sample of 2:4-lutidine showing the presence of γ-picoline. Curve *D*: analysis of a commercial sample of collidine showing the presence of 2:4-lutidine. In addition, the broadness of the collidine band indicates the presence of two further components.

Table 4. *Relative retention volumes of pyridine homologues*

(Temperature, 137°; values relative to that of pyridine, taken as 1.)

Column liquid phase	α-Picoline	β-Picoline	γ-Picoline	2:6-Lutidine	2:4-Lutidine	2:4·6-Collidine
DC 550	1·39	2·3	2·24	1·94	2·82	4·30
Lubrol MO	1·50	2·3	2·34	2·15	3·40	4·85
Liquid paraffin	1·75	2·4	2·31	2·80	4·00	6·35

EXPERIMENTAL

Preparation of columns. The columns were of the size (4 ft. long and 4 mm. internal diameter) described by James & Martin (1952). The kieselguhr used was prepared as described by James *et al.* (1952). The liquid phases of liquid paraffin, 'Lubrol MO' (Imperial Chemical Industries Ltd.) and DC 550 (Midland Silicones Ltd.) were added to the kieselguhr in the proportion of 3 g. of liquid phase to 7 g. of kieselguhr.

Procedure. The amines were added to the columns with a micropipette either in ethanolic solution (33 %, w/w) or as the pure dry amine. The titrations were carried out using the automatic recording burette of James & Martin (1952) filled with 0·04 N-H_2SO_4. The burette has been modified to take two burette tubes held parallel to one another, one containing dilute acid, the other dilute alkali. While one is in operation connected to the titration cell, the other is con-

nected only to its reservoir. In this way either bases or acids may be titrated without having to empty and refill the burette. The indicator used was methyl red in saturated aqueous solution. For further experimental details the two earlier papers should be consulted.

SUMMARY

1. The separation and micro-estimation of a series of aliphatic primary, secondary and tertiary amines is described.

2. A straight-line relationship is obtained when the logarithms of the retention volumes are plotted against the number of carbon atoms for each type of primary, secondary and tertiary amine. By means of this relationship the relative retention volume of any amine may be predicted provided the retention volume of any other member in the correct homologous series is known.

3. The utilization of columns having polar and non-polar liquid phases to determine whether an unknown amine is primary, secondary or tertiary is described. A plot of the relative retention volumes of primary, secondary and tertiary amines on paraffin against those obtained with 'Lubrol MO' liquid phases shows the values for each type of amine to fall on straight lines of different slope.

4. Out of 27 amines containing from 1 to 12 carbon atoms only two pairs (*iso*- and *sec*.-butylamine; di*iso*- and di-*sec*.-butylamine) cannot be separated on 4 ft. columns.

5. The separation of the homologues of pyridine is described as a suitable method for the approximate standardization of pyridine bases for chromatographic use.

Thanks are due to Dr A. J. P. Martin, F.R.S., for much valuable advice and discussion. Appreciation is also expressed to the National Coal Board (Eastern Region) and Messrs Yorkshire Tar Disillers Ltd. for gifts of pyridine homologues.

REFERENCES

James, A. T. & Martin, A. J. P. (1952). *Biochem. J.* **50**, 679.

James, A. T., Martin, A. J. P. & Smith, G. Howard (1952). *Biochem. J.* **51**, 323.

Studies in Immunochemistry

11. THE ISOLATION AND PROPERTIES OF THE HUMAN BLOOD-GROUP H SUBSTANCE

By E. F. ANNISON and W. T. J. MORGAN
The Lister Institute of Preventive Medicine, London, S.W. 1

(*Received* 11 *January* 1952)

The different sera of human and animal origin which bring about the agglutination of human O erythrocytes strongly, whilst agglutinating cells belonging to other blood groups less readily or not at all, are generally referred to as 'anti-O' sera. The isolation from human red cells of a material possessing the capacity to neutralize the agglutinating action of these sera on O cells was first reported by Hallauer (1934) and subsequently by Stepanov, Kuzin, Markageva & Kosyakov (1940). Witebsky & Klendshoj (1941) described the isolation of a small quantity of serologically active material from the gastric juice of group O persons. The homogeneity of the substances was in no instance established, but the results were important in that they demonstrated that serologically specific materials of carbohydrate nature could be obtained from the erythrocytes and gastric juice of group O persons. Landsteiner & Harte (1941) likewise isolated the so-called O substance from the saliva of individuals belonging to group O and showed that the material was largely carbohydrate in nature and could not be distinguished chemically from that obtained from the saliva of persons belonging to groups A and B.

Following the demonstration by Morgan & Van Heyningen (1944), that ovarian cyst fluids obtained from women secreting their specific blood-group substances in a water-soluble form are a convenient and potent source of these materials, Morgan & Waddell (1945) isolated from this source a polysaccharide-amino-acid complex which possessed the so-called O specific character. The material obtained was not electrophoretically homogeneous, but nevertheless a preliminary examination of the substance was undertaken and it was found to possess a laevorotation with $[\alpha]_{5461} - 22°$, to contain 5·9 % nitrogen and to yield, after acid hydrolysis, 43 % of reducing sugars, expressed as glucose, and 33 % of hexosamine, as glucosamine base. Hydro-

lysis of the material with strong acid revealed that 43 % of the total nitrogen was present as amino-acid nitrogen and that the nitrogen of the liberated α-amino groups accounted for at least 81 % of the total nitrogen of the O substance. The sera of rabbits immunized with this so-called O substance were found to contain, after absorption with A_1B cells, quite potent antibody reactive against O and A_2 cells.

It is now recognized that the so-called anti-O sera detect a substance which is not a specific product of the activity of Bernstein's *O* gene, but that they react with a material which is present to a variable extent on the surface of the erythrocytes of the majority of human subjects. A substance which is probably identical serologically with that which occurs on the red-cell surface is found in the secretions of about 75 % of all persons and, as was clearly demonstrated by Schiff & Sasaki (1932), is present in the secretions of individuals who belong to blood group A_1B, i.e. in the secretions of persons who do not possess an *O* gene. These and other observations would seem to indicate that the so-called O substance described in the literature is not a specific product of the *O* gene, and to avoid confusion Morgan & Watkins (1948) proposed that the material be called H substance in place of O substance.

Detailed procedures for the isolation of certain of the human blood-group factors from ovarian cyst fluids have already been reported (Morgan & Waddell, 1945; Aminoff, Morgan & Watkins, 1950; Annison & Morgan, 1952), and essentially similar methods are now described for the isolation and characterization of the H substance from the same source.

EXPERIMENTAL

The cyst fluids studied were obtained from group O individuals and those fluids were selected which were rich in H substance. The fluids chosen usually possessed a relatively low concentration of the Lewis blood-group substance Le^b and, in certain specimens, of Le^a substance also (Grubb & Morgan, 1949; Grubb, 1951).

Materials and methods

Cyst fluids. These materials were usually dried from the frozen state and stored at 0° or −10° until required. Cyst fluids preserved in their native state were kept under toluene at 0°.

Serological activity. The agglutination tests employed to measure the activity of the preparations of H substance were carried out as described by Morgan & Van Heyningen (1944), and all the different anti-H sera available (Annison & Morgan, 1950) were also used at one time or another as control reagents. When human anti-H sera were used, much smaller volumes were taken and a technique similar to that described by Grubb & Morgan (1949) for Le^a inhibition tests was employed. A 'standard' preparation of H substance of human origin was included in all tests.

Analytical methods. The procedures employed to examine the H substance were the same as those used in similar studies with the blood-group A and Le^a substances (Aminoff & Morgan, 1948; Aminoff *et al.* 1950; Annison & Morgan, 1952). C, H and N analyses were made by Weiler and Strauss, Oxford. Optical rotations were measured at room temperature, approx. 20°.

The isolation of the H *substance*

Preliminary experiments using cyst fluid no. 93 indicated that when the dried fluid was extracted with 90 % (w/v) phenol-water solution the serologically active substance remained insoluble in the liquid phenol. In view of the ease with which this procedure eliminated most of the contaminating protein in one step, the remainder of the dried cyst fluid (92 g.; N, 12·9 %), which possessed about 5 % of the specific activity of the 'standard' H preparation, was similarly extracted. The phenol-insoluble residue was suspended in water and dialysed for several days at 0–2° and the water-insoluble component thoroughly extracted with water. The aqueous solution and the combined washings were concentrated *in vacuo* and dried from the frozen state to yield a product (8·56 g.; N, 5·9 %) which was as active as the laboratory 'standard' H substance. A sample of the water-insoluble residue was dissolved by adding a little alkali to an aqueous suspension of the material maintained at 0°. Serological tests indicated that this material (6·6 g.; N, 7·2 %) possessed rather less than half the activity of the 'standard' H solution. The remainder of the water-insoluble material was subsequently rendered water-soluble by pepsin treatment as described later in this paper.

The absence of a precipitate on the addition of 10 % (w/v) trichloroacetic acid to the water-soluble material (N, 5·9 %) indicated that the preparation was substantially free from extraneous protein, but in order to remove the remaining traces of protein, deproteinization (Sevag, 1934) was applied. The best conditions for the removal of protein were found by preliminary experiments and applied as follows: a solution (0·5 %) of the substance in 0·1 M-sodium acetate, pH 4·6, was vigorously stirred in 250 ml. centrifuge pots with an equal volume of a 1:1 mixture of $CHCl_3$ and amyl alcohol for about 30 min. The two liquid phases were then separated by centrifugation, and the supernatant fluid removed. The denatured protein which collected at the solvent interface was treated with an equal volume of fresh buffer, the aqueous layer recovered and added to the first aqueous phase. The denatured material was collected by pouring the $CHCl_3$-amyl alcohol layer and the material suspended in it into excess ethanol, and separating the precipitated material by centrifugation. The specific substance was treated three times with the organic solvent mixture. The results shown in Table 1 indicate that this treatment was effective in deproteinizing the mucoid solution, although a good deal of the specific substance separated with the denatured protein.

The deproteinized material (Table 1, fraction *e*) was examined electrophoretically at pH 8·0, and was found to show some inhomogeneity. This partially purified H substance was subsequently fractionated from aqueous solution at 37° with Na_2SO_4. Powdered anhydrous Na_2SO_4 was slowly added, with stirring, to a 2 % solution of the H substance (4·1 g.) to yield a concentration of 22 % (w/v) Na_2SO_4. In order to maintain more precise control over the precipitation, further Na_2SO_4 beyond this amount was added as a saturated solution. Four fractions were collected,

the last of these being the material remaining in solution after saturation with Na_2SO_4 had been reached. The largest fraction was redissolved in water and the fractional precipitation with Na_2SO_4 repeated. A large active fraction was again thrown down over a narrow range of Na_2SO_4 concentration. The results of these fractionations are shown in Table 2. A sample of the purified substance (Table 2, fraction 2*b*) dissolved in water to give a fairly viscous opalescent solution. Electrophoretic analysis of this material at pH 8·0 again demonstrated some inhomogeneity. The subsequent isolation of electrophoretically homogeneous blood-group substances from these materials suggested the use of the same technique with the H substance.

A small-scale experiment revealed that some 30–40 % of the partially purified product obtained by Na_2SO_4 fractionation was rendered phenol-soluble by the trichloroacetic acid treatment, and this material, after precipitation from phenol solution with ethanol, was found to possess a somewhat higher activity than the standard H substance. The whole of the available substance was therefore treated with

Table 1. *Removal of protein from partially purified* H *substance by treatment with* $CHCl_3$*-amyl alcohol mixture*

(The serological activity of a material in this table and in the following tables is expressed in terms of the activity of a standard solution of the H substance.)

Nature of fraction	Yield (g.)	Specific rotation $[\alpha]_{5461}$	N content (%)	Serological activity (% of standard preparation)
(*a*) 1st	1·92	*	6·35	100
(*b*) 2nd } Material separating at interface	1·10	−47°	6·3	100
(*c*) 3rd	0·35	−35°	5·85	100
(*d*) 1st	—	−35°	5·4	100
(*e*) 3rd } Material soluble in aqueous phase†	4·53	−32°	5·2	100

* Too turbid for accurate polarimetric observation. † Small sample only examined.

Table 2. *Fractionation of partially purified* H *substance with* Na_2SO_4

Fraction	Precipitation level (% (w/v) Na_2SO_4)	Yield (g.)	Specific rotation $[\alpha]_{5461}$	N content (%)	Serological activity (% of standard preparation)
1 (*a*)	0–24·6	0·10	*	4·45	100
(*b*)	24·6–25·8	3·05	−20°	5·6	100
(*c*)	25·8–27·0	0·32	−35°	4·45	100
(*d*)	27·0–Excess	0·24	−30°	4·4	50
(*e*)	Material in final supernatant fluid	0·17	−29°	4·4	50
	Further fractionation of 1 (*b*)				
2 (*a*)	0–24·5	0·07	*	5·0	100
(*b*)	24·5–26·0	2·83	−24°	5·65	100
(*c*)	Material soluble above 26·0	0·09	−32°	4·9	100

* Too turbid for polarimetric observation.

Table 3. *Fractionation of partially purified* H *substance from* 90 % *phenol with ethanol*

Fraction	Precipitation level (% (v/v) EtOH)	Yield (g.)	Specific rotation $[\alpha]_{5461}$	N content (%)	Serological activity (% of standard solution)
1 (*a*)	Phenol-insoluble material	1·41	−48°	5·05	100
(*b*)	0–10·0	1·15	−37°	5·3	150
(*c*)	10–excess	0·15	−30°	6·3	100
	Further fractionation of 1 (*b*)				
2 (*a*)	Phenol-insoluble	0·29	−43°	5·2	100
(*b*)	0–3·5	0·37	−33°	5·3	150
(*c*)	3·5–7·0	0·23	−29°	5·3	150
(*d*)	7·0–10·0	0·20	−30°	5·3	150
(*e*)	10–excess	0·01	−36°	5·4	150

The specific substance at this stage of purification was insoluble in phenol, but earlier work showed that both the A substance (Aminoff *et al.* 1950) and the Le^a substance (Annison & Morgan, 1952) became in part phenol-soluble on treatment with 5 % trichloroacetic acid at 0° for a short time. 5 % (w/v) trichloroacetic acid. A precipitate did not form, showing the absence of appreciable quantities of free protein. The solution was neutralized, dialysed, and the material was recovered by drying from the frozen state. The dry substance was then exhaustively extracted with 90 %

phenol, and the soluble material divided into two fractions by the addition of ethanol to the phenol solution. The ethanol level was adjusted to 10% (v/v), by the addition of 1:1 phenol-ethanol mixture and the material thrown out of solution was collected by centrifugation. The supernatant solution was treated with several times its volume of ethanol, and the precipitate formed was collected. The phenol-insoluble residue and the two phenol-soluble products were washed free from phenol with ethanol, dialysed at 0° against water and dried from the frozen state. The analytical figures and serological activities of these fractions are shown in Table 3.

The fraction precipitated over the range 0–10% ethanol (1*b*) was resuspended in liquid phenol, when a small amount of it failed to dissolve after shaking for some hours with successive portions of fresh phenol. The soluble substance was fractionally precipitated from solution by slowly adding increasing amounts of a 1:1 (v/v) 90% phenol-ethanol mixture. Three major fractions separated between the levels 0 and 10% (v/v) ethanol: only a trace of material was precipitated by the addition of excess ethanol. The results of the final fractionation are included in Table 3. The analytical figures of fractions *b*, *c* and *d* suggested that the phenol-soluble material was not grossly inhomogeneous chemically and these fractions were therefore united. Electrophoretic and ultracentrifugal analysis of this product at pH 4·0 and 8·0 showed the presence of only one component (see Addendum to this paper).

half the activity of the standard H substance, was extracted with 90% phenol when rather more than half of the material dissolved. The phenol-insoluble residue (Fig. 1, 1*b*) dissolved in water to form a highly viscous, turbid solution, which possessed not more than 25% of the activity of the standard H solution. The phenol-soluble material was fractionally precipitated from solution with ethanol, and the fractionation repeated several times (Fig. 1). Each fraction collected was washed free from phenol with ethanol, dissolved in water, dialysed at 0°, and dried from the frozen state.

The final product precipitated from phenol over the range 0–10% ethanol (Fig. 1, 4*b*) was split into four parts by further fractionation from phenol between narrower limits of ethanol concentration. The hexosamine content and reducing-sugar value relative to glucose of each fraction after hydrolysis with 0·5 N-HCl at 100° for 16 hr. was estimated, in addition to the analytical figures usually determined, as shown in Table 4. The analytical results suggested that the starting material 4*b* was essentially homogeneous, and in consequence fractions 5*a*, *b*, *c* and *d* (Table 4) were combined. The product was examined in the Tiselius electrophoresis apparatus at pH's 8·0 and 4·0, when the presence of only one component was demonstrated. Ultracentrifugal analysis also failed to show that the preparation was inhomogeneous.

The fucose content (13·1%) of this preparation, which was isolated by means of peptic digestion, was found to be slightly lower than that of the first preparation (14·2%) (Table 3, 2*b*, *c* and *d*). The former preparation was also shown

Table 4. *Fractionation of* H *substance from solution in* 90% *phenol by ethanol*

(H substance used was material derived from fraction 4*b* in Fig. 1.)

Fraction	Precipitation level	Yield (g.)	$[\alpha]_{5461}$	N (%)	Hexosamine (%)	Reduction (%)	Serological activity (% of standard solution)
5 (*a*)	0–3·0	0·38	−26°	5·3	29·6	53	150
(*b*)	3·0–5·5	0·73	−31°	5·25	30·0	53	150
(*c*)	5·5–7·5	0·57	−32°	5·3	29·1	54	150
(*d*)	7·5–10·0	0·11	−28°	5·35	29·7	53	150

Pepsin treatment of the H *substance*

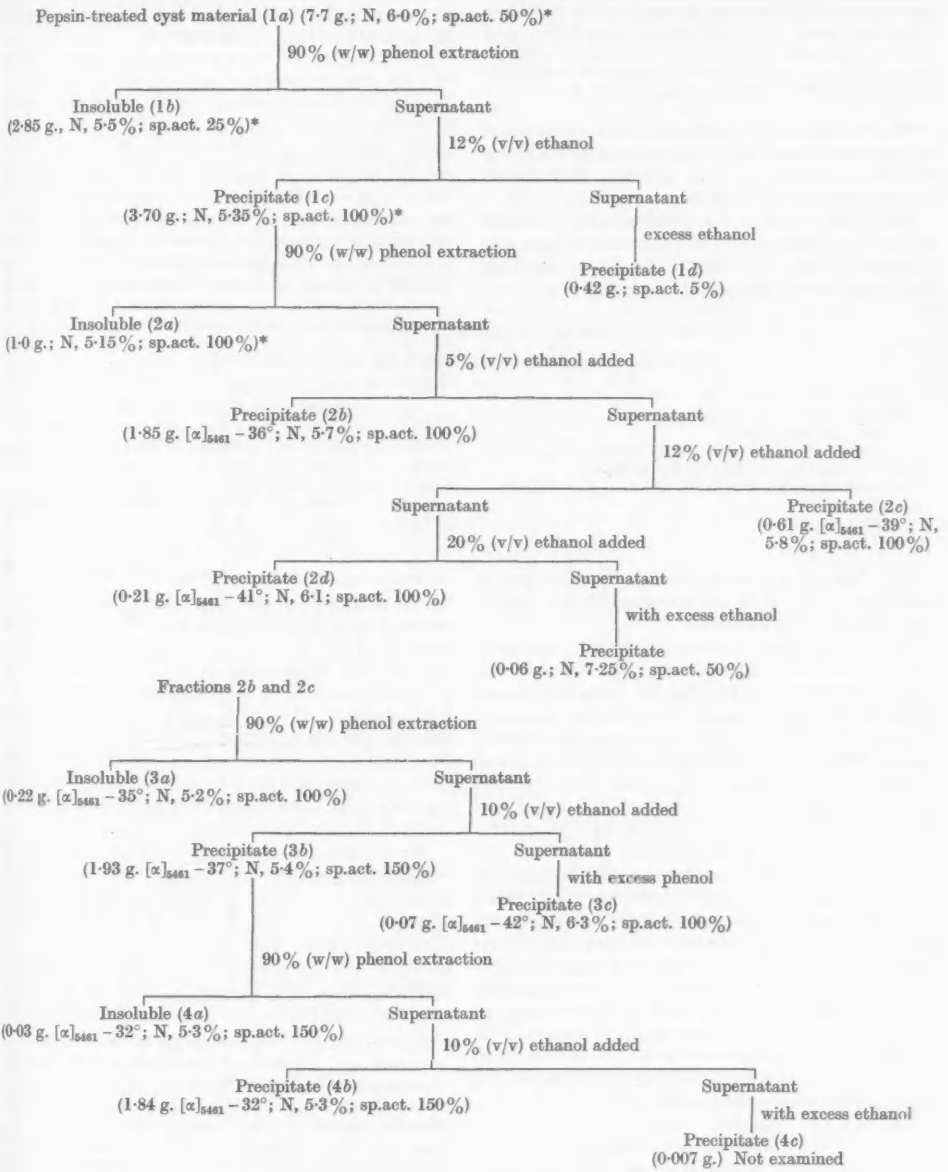
A considerable portion of the specific material present in the crude cyst, the whole of which was originally soluble in water, was found, after drying from the frozen state and treatment with phenol, to be insoluble in water. A possible interpretation of this phenomenon is that part of the group substance present in the cyst was bound to protein which was denatured and thus made insoluble. An attempt was therefore made to liberate this non-available group substance by digesting the insoluble material with pepsin. A small amount of the water-insoluble material was suspended with a trace of crystalline pepsin in 0·2 M-HCl-citrate, pH 2·6, at 37° for 48 hr. The substance passed completely into solution and after dialysis at 0° was dried from the frozen state to yield a water-soluble product possessing about half the serological activity of the standard H preparation. The whole of the water-insoluble material, together with a number of specimens similarly contaminated with protein (11·6 g.) which arose as side fractions in other purification procedures, was digested with 2 mg. of crystalline pepsin under similar conditions. The solution obtained was thoroughly dialysed at 0°, and dried from the frozen state. The main product (Fig. 1, 1*a*, 7·7 g.; N, 6·0%) which possessed about to possess a slight capacity to precipitate with anti-pneumococcal type XIV serum, a property not shown by the other material. These results suggest that a slight degradation had taken place during peptic digestion and the material was accordingly used as the starting material from which the sugar components, after they had been identified in the best H preparation, were isolated.

Test for homogeneity

The homogeneity of the main preparation (Table 3, fractions 2*b*, *c* and *d*) was studied by the following procedures.

(1) *Fractional solubility test.* The ready and extreme solubility of the H substance in water, with the formation of highly viscous solutions, prevented the application of the usual equilibrium method of solubility test. A fractional solubility test of the type employed for the human A substance (Aminoff *et al.* 1950) was therefore used.

The preparation (650 mg.) was shaken in a 50 ml. centrifuge pot with successive portions (about 20 ml.) of 32% (v/v) ethanol-water. The suspension was shaken for about


Pepsin-treated cyst material (1a) (7·7 g.; N, 6·0%; sp.act. 50%)*
90% (w/w) phenol extraction
Insoluble (1b) (2·85 g., N, 5·5%; sp.act. 25%)*
Supernatant
12% (v/v) ethanol
Precipitate (1c) (3·70 g.; N, 5·35%; sp.act. 100%)*
Supernatant
excess ethanol
Precipitate (1d) (0·42 g.; sp.act. 5%)
90% (w/w) phenol extraction
Insoluble (2a) (1·0 g.; N, 5·15%; sp.act. 100%)*
Supernatant
5% (v/v) ethanol added
Precipitate (2b) (1·85 g. [α]5461 −36°; N, 5·7%; sp.act. 100%)
Supernatant
12% (v/v) ethanol added
Supernatant
Precipitate (2c) (0·61 g. [α]5461 −39°; N, 5·8%; sp.act. 100%)
20% (v/v) ethanol added
Precipitate (2d) (0·21 g. [α]5461 −41°; N, 6·1; sp.act. 100%)
Supernatant
with excess ethanol
Precipitate (0·06 g.; N, 7·25%; sp.act. 50%)
Fractions 2b and 2c
90% (w/w) phenol extraction
Insoluble (3a) (0·22 g. [α]5461 −35°; N, 5·2%; sp.act. 100%)
Supernatant
10% (v/v) ethanol added
Precipitate (3b) (1·93 g. [α]5461 −37°; N, 5·4%; sp.act. 150%)
Supernatant
with excess phenol
Precipitate (3c) (0·07 g. [α]5461 −42°; N, 6·3%; sp.act. 100%)
90% (w/w) phenol extraction
Insoluble (4a) (0·03 g. [α]5461 −32°; N, 5·3%; sp.act. 150%)
Supernatant
10% (v/v) ethanol added
Precipitate (4b) (1·84 g. [α]5461 −32°; N, 5·3%; sp.act. 150%)
Supernatant
with excess ethanol
Precipitate (4c) (0·007 g.) Not examined

* Aqueous solutions too turbid for polarimetric observation.

Fig. 1. The fractionation of pepsin-treated H substance from solution in 90% phenol by ethanol. (The serological activities of the materials given in this figure are expressed in terms of the activity of a standard solution of this H substance, shown as sp.act., x%.)

1 min., centrifuged to separate the liquid and solid phases, the supernatant fluid decanted, fresh solvent added, and the procedure repeated. Five fractions, including the final solid residue, were thus obtained. The serological activity, specific rotation, N content, reducing power and hexosamine content of each fraction are recorded in Table 5.

The results obtained indicated that the material was probably homogeneous chemically and contained no material of very different composition from that of the most active material.

(2) *Electrophoresis.* An electrophoretic analysis of the H substance at pH's 4·0 and 8·0 gave no evidence of inhomogeneity. Details are given in the Addendum to this paper.

anti-H serum was observed to occur with an equal volume of a dilution of 1 in 5×10^5 of H substance, i.e. with about 1 μg. of the material.

The H substance, when tested at a concentration of 5 mg./ml., showed no capacity to inhibit two or three completely agglutinating doses of the following heterologous blood-group reagents: (1) human anti-A and human anti-B agglutinins; (2) rabbit anti-M and anti-N agglutinins; (3) anti-P agglutinins; (4) anti-*Rhesus* sera, consisting of anti-D, anti-C, anti-c and anti-E reagents, and (5) anti-Le^a and anti-Le^b agglutinins. Some inhibition of human anti-O serum (Morgan & Watkins, 1948) was observed, however, although comparative tests with

Table 5. *Fractional solubility test for homogeneity on* H *substance*

(For details of fractionation see Table 3, 2*b*, *c* and *d*, and Text.)

Fraction	I	II	III	IV	V
Specific rotation $(\alpha)_{5461}$ (± 5)	−26°	−30°	−29°	−31°	−27°
(*a*) N (Kjeldahl) (%)	5·2	5·2	5·1	5·2	5·2
(*b*) Reduction (%) (calculated as glucose)	52·2	52·7	52·5	53·1	52·4
(*c*) Hexosamine (%) (calculated as base)	30·3	30·8	30·9	30·3	31·0
(*d*) Serological activity (% of standard preparation)	150	150	150	150	150
b/*a*	10·04	10·13	10·29	10·21	10·08
c/*a*	5·82	5·92	6·06	5·83	5·96
b/*c*	1·72	1·71	1·70	1·72	1·69

(3) *Ultracentrifugal examination.* The H substance was also examined in the ultracentrifuge. Details are given in the Addendum.

(4) *Quantitative immunochemical analysis.* Through the kindness of Prof. J. R. Marrack and Mr R. G. S. Johns the H substance from cyst 93 was examined by a quantitative precipitation technique similar to that described for A substance by Kabat, Baer & Knaub (1949). The antiserum employed was prepared by us in rabbits by means of an artificial antigenic complex made from the H substance and the conjugated protein component of the O somatic antigen of *Shigella shigae* (Morgan, 1943). The percentage of the glucosamine in the added H substance that was precipitated was calculated with allowance for solubility of the precipitate and for the apparent glucosamine content of the antibody, as done by Bendich, Kabat & Bezer (1946); allowance was also made for an effect of protein on estimates of glucosamine (Johns & Marrack, 1952). In the region of antibody excess the percentage of glucosamine precipitated, calculated in this way, was from 78 to 110. The full results of these experiments will be published in a separate paper.

Properties of the H *substance*

Immunological properties. The serological activity of the H substance was measured by determining the minimum amount necessary to inhibit the agglutinating action on O cells of anti-H sera of human and animal origin. The inhibition of two to three completely agglutinating doses of human an inhomogeneous preparation of O substance obtained from human erythrocytes of group O suggested that the O substance content of the H preparation was equivalent to not more than a few per cent of the preparation from O stroma. The H preparation showed no capacity to inhibit the haemolysis of sheep red cells by rabbit anti-A erythrocyte serum in the presence of complement. Samples of the material were tested after treatment with N-acetic acid at 100° for 1–6 hr., but in no instance was there any evidence for the development of 'Forssman' activity.

Earlier investigations suggested that a partially purified preparation of the H substance isolated from human ovarian-cyst fluid was antigenic when injected into rabbits (Morgan & Waddell, 1945). This conclusion does not seem to hold for the preparations of H substance isolated during the present work, judging from the results of similar experiments with the more highly purified material.

A group of six rabbits, selected for the low reactivity of their sera with A and B cells, was given, at 3- to 4-day intervals, three intravenous doses each of 0·1 mg. of H substance dissolved in saline. The rabbits were bled 1 week after the last dose. No significant increase in the agglutination titre of the sera against O cells with respect to that of the normal bleedings was found. The rabbits were now given a further three intravenous doses, each of 0·1 mg. of the H substance, under the same conditions as before. The anti-O cell titre of each of the resulting sera, however, was not appreciably higher than that of the normal bleedings.

A similar group of six rabbits was given, under the same conditions, three doses, each of 0·05 mg., of an artificial

antigen made by coupling the same specimen of H substance with the conjugated protein component of the O somatic antigen of *S. shigae*. This antigen was prepared by the method described for the corresponding artificial A antigen (Morgan, 1943). The average anti-O cell agglutinin titre increased to about 2000, a 100-fold enhancement above the normal titre. The results indicated that under the conditions of test in rabbits the H substance is not antigenic, or only very weakly so, whereas it is readily converted into a powerful antigen by combination with the conjugated protein component of *S. shigae*.

The preparation of the H substance which had not been treated with pepsin failed to give a precipitate when tested with horse anti-pneumococcal type XIV serum, and in this respect differed from the H-active materials isolated by Kabat and his co-workers (see Kabat, 1949). The H preparation isolated by the use of pepsin, however, showed a weak reactivity when tested at a dilution of 1:1000 with the pneumococcus type XIV antiserum. The type XIV polysaccharide gave a heavy precipitate when mixed with its homologous serum under similar conditions.

The H substance was examined by Dr J. McCrea for its capacity to inhibit the agglutination of fowl cells by the heated Lee (type B) influenza virus. The materials were found to be inactive. The high activity of a specimen of purified human blood-group H (so-called 'O') substance reported by Burnet, McCrea & Anderson (1947) must be attributed to an active material contaminating the preparation examined.

Physical properties. The relative viscosity (η) of a 0·5 % solution of the H substance in 0·85 % saline was 1·31. The mean value of the specific rotation of the most active preparations of the H substance, was $[\alpha]_{5461} - 30 \pm 3°$ in water (c, 1·0).

The ultraviolet absorption of the H substance (0·1 % in 0·85 % sodium chloride) was examined in a Hilger quartz spectrophotometer (Uvispek) using optical cells 2 cm. deep. The absorption curve (Fig. 2) shows that no absorption maxima occur between 220 and 300 mμ.

Chemical properties. A precipitate was not formed when a 2 % aqueous solution of the H substance is treated with any of the usual protein precipitants. The biuret, ninhydrin, xanthoproteic, Millon, Hopkins-Cole, Seliwanoff and Bial tests were negative. Ehrlich's diazosulphanilic acid test was faintly positive as was Sakaguchi's test for arginine. The addition of alkaline copper sulphate to a solution of the H substance, as in the biuret test, gave rise to a voluminous precipitate.

The analytical figures for the pooled specimen of H substance (Table 3, fractions 2*b*, *c* and *d*) isolated from cyst no. 93 are: C, 41·4; H, 6·9; N, 5·3 % (Kjeldahl). The phosphorus content is less than 0·02 %, and the sodium fusion test for sulphur indicates that this element is not present in appreciable amounts.

The acetyl content of the H substance is 8·7 % (6 hr. hydrolysis) and is in excess of that required for the *N*-acetylhexosamine calculated to be present from the total hexosamine value. The distillates obtained from the acetyl determinations were neutralized and were used to identify the volatile organic acid as an *S*-benzylthiouronium salt (Donleavy, 1936). The *S*-benzylthiouronium salt obtained after recrystallization from 80 % ethanol melted at 134° and gave a mixed m.p. of 136° with an authentic specimen of *S*-benzylthiouronium acetate (m.p. 136°).

The total fucose content of the H substance was rather more than 14 %. Under the conditions used for the determination of *N*-acetylglucosamine (Morgan & Elson, 1934; Aminoff, Morgan & Watkins, 1952) the H substance gave a colour with Ehrlich's reagent. The maximum colour developed

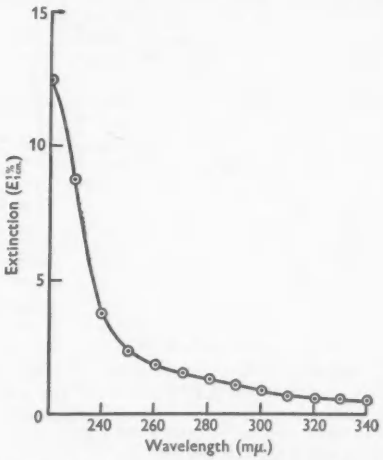


Fig. 2. The ultraviolet absorption spectrum of H substance (0·1 % solution in 0·85 % NaCl).

by the H substance after heating at 100° for 12 min. with dilute sodium carbonate at pH 10·8 (optimal heating conditions), was equivalent to about 7·5 % (unextrapolated value) of the colour given by an equal weight of *N*-acetylglucosamine. *N*-acetylglucosamine requires 4 min. heating to develop its maximum colour under the same conditions.

The H substance after heating with dilute sodium carbonate for 16 min. at 100° was dialysed through well washed cellophan against distilled water at 0°. The diffusate gave an immediate colour with Ehrlich's reagent, whereas the indiffusible residue gave no appreciable colour (cf. King & Morgan, 1944; Morgan, 1946 Annison & Morgan, 1952).

Oxidation of the H *substance with periodate*

The rate of reduction of periodate by the H substance at pH's 5·0 and 7·3 was measured by the

techniques employed by Aminoff & Morgan (1951) in similar experiments with the human A substance.

Oxidation at pH 5·0. A solution (0·1 %) of the H substance in 0·1 M-acetate, pH 5·0, was treated with 0·005 M-HIO_4. The rate of reduction of periodate and the loss of biological activity of the substance were determined. The results (Fig. 3) indicate that the rate of reduction of periodate at pH 5·0 by H substance is similar to that found for the A and Le^a substances. The biological activity of the H substance, however, is destroyed much more rapidly than the activity of the A and Le^a substances under similar conditions. The analytical results, corrected for over-oxidation by extrapolating the uptake curve to zero time, show that 1 mg. of the H substance reduces $0{\cdot}277 \times 10^{-5}$ mole of HIO_4, and hence about 362 g. of H substance reduces 1 mole of HIO_4.

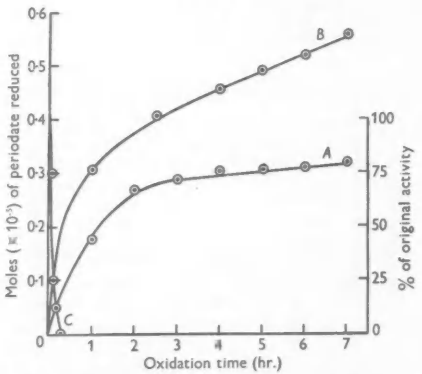


Fig. 3. The rate of reduction of periodate by H substance at pH 5·0 (curve *A*) and pH 7·3 (curve *B*). The resulting inactivation at pH 5·0 as determined by the capacity of the oxidized material to inhibit the agglutination of O cells by anti-H agglutinin (curve *C*).

Oxidation at pH 7·3. The rate of reduction of periodate is greater at pH 7·3 than it is at pH 5·0 (Fig. 3). The uptake curve shows a less definite inflexion after 2 hr. and the periodate consumption continues steadily for several days. The serological activity of the substance is lost at approximately the same rate as at pH 5·0 (Fig. 3).

Hydrolysis of the H *substance by acid*

The H substance was hydrolysed in sealed glass ampoules at 100° with (*a*) 0·5 N-HCl, (*b*) 6 N-HCl and (*c*) N-acetic acid, as described for the Le^a substance (Annison & Morgan, 1952).

The course of hydrolysis with 6 N-HCl was followed by measuring the α-amino N and the α-amino-acid N liberated. The results (Fig. 4) show that the liberation of α-amino N reaches a maximum in about 4 hr. and then represents 88 % of the total N of the H substance, whereas the α-amino-acid N after 16 hr. hydrolysis is equivalent to about 41 % of the total N. The α-amino N value includes the N liberated from the amino sugars set free during hydrolysis.

The liberation of reducing sugars and hexosamines was followed by an examination of the products of hydrolysis with 0·5 N-HCl. The results (Fig. 5) show that the maximum liberation of reducing sugars, 54 % expressed as glucose, and of hexosamines, 31 % expressed as glucosamine base, occur at about the same time (6 hr.).

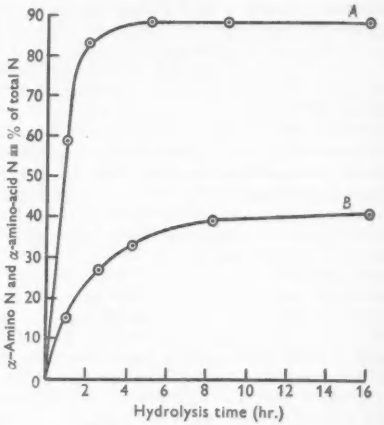


Fig. 4. Hydrolysis of H substance by 6 N-HCl at 100° as determined by the liberation of α-amino groups (curve *A*), and α-amino-acids (curve *B*).

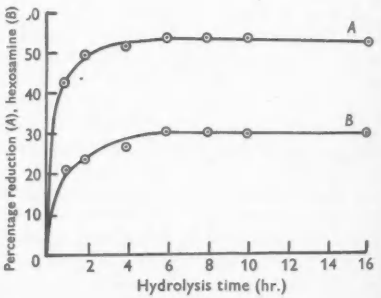


Fig. 5. Hydrolysis of H substance by 0·5 N-HCl at 100° as determined by (*A*) reducing power, expressed as glucose and (*B*) hexosamine liberated, expressed as glucosamine base.

The H substance was hydrolysed with N-acetic acid at 100°, and the rate and extent of liberation of fucose, reducing sugars and *N*-acetylhexosamine residues determined. The results (Fig. 6) show that a maximum value of about 13 % fucose determined as acetaldehyde after oxidation with periodate is obtained after 20 hr. hydrolysis and a maximum value for reducing sugars is reached at about the same time. There is an appreciable increase in the amount of '*N*-acetylhexosamine' colour given by the H substance during the first 2 hr. of the hydrolysis period. After this time the original value of 7·5 % expressed as *N*-acetylglucosamine, reaches a maximum value of 8·8 % and then decreases.

Treatment with N-acetic acid at 100° rapidly inactivates the H substance and at the same time induces the formation of a product which possesses the property of reacting with horse anti-pneumococcal type XIV serum.

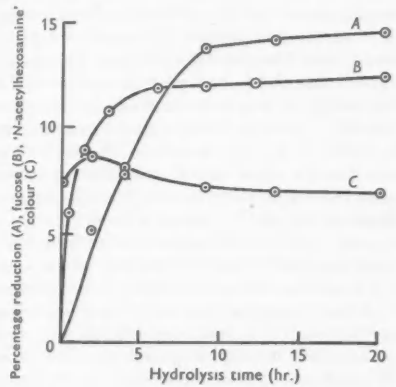


Fig. 6. Hydrolysis of the H substance by N-acetic acid at 100° as determined by (*A*) reducing power, expressed as glucose, (*B*) fucose, determined as acetaldehyde after oxidation with periodate and (*C*) '*N*-acetylhexosamine' colour.

Chromatographic analysis of the H *substance*

A detailed investigation of the sugar and amino-acid components of the H substance was made by the usual techniques of partition chromatography on paper. The experimental procedures employed were the same as those described for similar work with the Le^a substance (Annison & Morgan, 1952).

Amino-acids. Two-dimensional paper chromatographic analysis of 6N-HCl hydrolysates of the H substance revealed that the amino-acid content of the material was indistinguishable from that of the A and Le^a substances (Aminoff *et al.* 1950; Annison & Morgan, 1952). The amino-acids identified on paper were as follows: lysine, arginine, aspartic acid, glutamic acid, glycine, serine, alanine, threonine, proline, valine and leucine (isoleucine).

Amino sugars. The presence of chondrosamine, in addition to glucosamine in the A substance, was first shown by Aminoff & Morgan (1948). Similar experiments established that both amino sugars were components of the H substance; similar findings have been reported for the Le^a substance (Annison & Morgan, 1952).

Sugars. D-Galactose and L-fucose were shown to be components of the H substance as described by Annison & Morgan (1952) for the Le^a substance.

Isolation of the component sugars and amino sugars

Sugars. The H substance (500 mg.) was hydrolysed with 0·5N-HCl and the product, after neutralization, run on a cellulose column according to the general procedure of Hough, Jones & Wadman (1949), using the solvent system 1:1 *n*-butanol/2% (v/v) acetic acid-water.

L-*Fucose diphenylhydrazone.* The fucose obtained from the column was dissolved in water (2 ml.), and glacial acetic acid (0·2 ml.) and freshly distilled diphenylhydrazine (60 mg.) added. The solution was allowed to stand overnight in the dark and at room temperature and was then cooled, when 30 mg. of needle-shaped crystals separated. The derivative was twice recrystallized from 95% (v/v) ethanol and 17 mg. of material melting at 200° were finally obtained. Mixed m.p. with authentic fucose diphenylhydrazone (Muther & Tollens, 1904) remained unchanged. (Found: C, 66·1; H, 6·8; N, 8·6. Calc. for $C_{18}H_{22}O_4N_2$: C, 64·7; H, 6·7; N, 8·5%.) Optical rotation $[\alpha]_D$ −14° in pyridine (*c*, 1).

D-*Galactose* α-*methylphenylhydrazone.* The fractions issuing from the column which contained galactose were evaporated to dryness *in vacuo* at about 30°. The residue was dissolved in water (2 ml.), and freshly distilled α-methylphenylhydrazine (0·1 ml.), ethanol (2 ml.) and 10% (v/v) acetic acid (0·1 ml.) were added. The mixture was kept at 37° for 2 hr. and overnight at 0°. D-Galactose α-methylphenylhydrazone (54 mg.) separated, and after recrystallization from 30% ethanol gave 48 mg. of needle-shaped crystals m.p. 191°. Mixed m.p. with authentic D-galactose α-methylphenylhydrazone 190°. (Found: C, 52·2; H, 6·9; N, 9·7. Calc. for $C_{13}H_{20}O_5N_2$: C, 54·9; H, 7·0; N, 9·9%.)

D-*Galactose* α-*methylphenylhydrazone pentaacetate.* Galactose α-methylphenylhydrazone (19 mg.) was suspended in 1 ml. of dry pyridine, and 0·2 ml. of acetic anhydride added at 0° according to the method of Wolfrom & Christman (1931). The mixture was allowed to stand overnight at 0°, and, with frequent shaking, at room temperature for a further 24 hr. The solution was then poured into excess chilled water and the white crystalline material which was thrown out of solution was collected and washed thoroughly with water. The material after recrystallization from 50% ethanol gave 29 mg. of D-galactose α-methylphenylhydrazone pentaacetate, m.p. 138–140° without decomposition. Mixed m.p. with authentic specimen of the pentaacetate remained unchanged. Specific rotation $[\alpha]_D$ +38±3° in 95% (v/v) ethanol (*c*, 1.)

2:4-*Dinitrophenyl* (*DNP*) *amino sugars.* The amino sugar components of the H substance were separated and identified as their 2:4-dinitrophenyl derivatives by partition chromatography on kieselguhr columns using the solvent system 30% amyl alcohol-$CHCl_3$/0·2M-potassium borate, pH 9·7 (Annison, James & Morgan, 1951). Two 100 mg. samples of the H substance isolated by the use of pepsin were hydrolysed for 16 hr. at 100° with 0·5N-HCl in an atmosphere of N_2. The products were condensed with 1-fluoro-2:4-dinitrobenzene and the resulting amino sugar derivatives separated and isolated. The results obtained with the two samples were: (1) Total yield of DNP amino sugars, 79%. DNP-glucosamine, 23·4 mg.; DNP-chondrosamine, 20·7 mg. Ratio, glucosamine: chondrosamine, 1:0·89. (2) Total yield of DNP amino sugars, 71%. DNP-glucosamine, 22·3 mg.: DNP-chondrosamine, 20·2 mg. Ratio, glucosamine: chondrosamine, 1:0·91. The yields of DNP amino-sugars were estimated colorimetrically.

N-2:4-*Dinitrophenyl*-D-*chondrosamine.* A part of the DNP-chondrosamine, obtained in Exps. 1 and 2 above, was pooled and recrystallized from acetone-water and then acetone. Yield, 9·2 mg., m.p. 178–180° (uncorr.), mixed m.p. with an authentic sample of DNP-chondrosamine, 179–181° (uncorr.). Specific rotation observed: $[\alpha]_{5461}$ +80±4° in 80% (v/v) ethanol (*c*, 1). (Found: C, 41·9; H, 4·7; N, 11·9. Calc. for $C_{12}H_{15}O_9N_3$: C, 41·7; H, 4·4; N,

12·2%.) $[\alpha]_{5461}$ +84±3° in 80% (v/v) ethanol (Annison *et al.* 1951).

N-2:4-*Dinitrophenyl*-D-*glucosamine.* A mixed specimen of DNP-glucosamine (61 mg.) obtained from a number of preparations of H substance was crystallized from acetone-water and from acetone-light petroleum (b.p. 69–80° range). Yield 12·2 mg., m.p. 197–200° (uncorr.). Optical rotation $[\alpha]_{5461}$ +66±4° (*c*, 1% in 80% (v/v) ethanol). (Found: C, 41·9; H, 4·3; N, 11·9. Calc. for $C_{12}H_{15}O_9N_3$: C, 41·7; H, 4·4; N, 12·2%.) $[\alpha]_{5461}$ +65±3° in 80% (v/v) ethanol (Annison *et al.* 1951).

Partial hydrolysis of the H *substance*

H substance (50 mg.) was hydrolysed with N-acetic acid at 100° for 48 hr. in sealed glass ampoules and the products were dialysed in a thoroughly washed cellophan bag for 7 days at 0° against frequent changes of distilled water. The diffusates were stored at 0° in the presence of a few drops of $CHCl_3$ until the dialysis was complete. The total diffusates were then mixed together, concentrated to small volume and dried from the frozen state. The non-diffusible contents of the cellophan bag were likewise dried. The diffusible material (about 19 mg.) was obtained as a hygroscopic glass, the non-diffusible material (16 mg.) as a light brown friable solid. Fucose, galactose, an *N*-acetylhexosamine and traces of glucosamine and chondrosamine were detected in the diffusate by paper chromatography. The solvents employed in this experiment did not resolve a mixture of *N*-acetylhexosamines and the precise identity of the material giving the *N*-acetylhexosamine spot was not elucidated. The developed chromatogram of the diffusate when sprayed with aniline hydrogen phthalate revealed the positions of fucose and galactose only. The $AgNO_3$-NH_3 reagent gave, in addition, three other spots due to slowly moving substances which presumably comprised partial hydrolysis products of the polysaccharide moiety of the H substance. Two of these three unidentified spots reacted also with the hexosamine reagent. The presence in the diffusate of an amino-acid or peptide which moved slowly in all the solvents used was revealed when the chromatograms were sprayed with ninhydrin. This spot and the other three unidentified spots were no longer present after the diffusate was hydrolysed further with 0·5N-HCl at 100° for 16 hr. Only fucose, galactose and glucosamine and chondrosamine, in what appeared to be approximately equal amounts, were detected in this hydrolysate. A part of the diffusate was hydrolysed further with 6N-HCl at 100° for 16 hr., and examined for amino-acids by two-dimensional paper chromatography. Traces of all the amino-acids present in the original material except leucine, proline and arginine were detected. The contents of the dialysis bag were examined chromatographically for sugars after hydrolysis with 0·5N-HCl. Fucose could not be detected in any of the chromatograms developed with the four solvent systems employed for sugar analysis. The presence of galactose and equal amounts of the two amino sugars was demonstrated. The qualitative composition of the indiffusible material in terms of amino-acids was found to be indistinguishable from that of the undegraded H substance.

DISCUSSION

The isolation of the blood-group H substance in an essentially homogeneous condition from human ovarian cyst fluids has allowed this immunologically important material to be studied and identified as a mucoid which possesses properties very similar to those of the other human blood-group substances already described.

The H substance sediments in the ultracentrifuge at a rate similar to that found for the blood-group A and Le^a substances under the same conditions (Kekwick, 1950, 1952) and the diffusion constants of the three materials are not grossly dissimilar. The particle weight of the H substance is of the order 320 000 and is thus somewhat larger than that found for the other two group substances. It may well be, however, that in their native state these materials have an even larger particle size. The specimens of H substance obtained possess a laevorotation of about −30°, and in this respect differ from the A substance prepared from cyst fluids which has a positive rotation. Kabat, Bendich, Bezer & Beiser (1947), however, record laevorotations for a large number of group A preparations isolated by them from human saliva, stomachs and amniotic fluids. The H substance contains the sugars L-fucose, D-galactose, D-glucosamine and D-chondrosamine and 11 amino-acids, and indeed is so far indistinguishable on qualitative grounds from the A and Le^a substances. Analysis of the H substance, however, reveals several differences in the quantitative make-up of the material compared with the other group substances. For example, the L-fucose content of the H substance appears to be not more than 14%, whereas this sugar is contained in the corresponding A mucoid to the extent of at least 18%. Similarly, the ratio of glucosamine to chondrosamine in the Le^a substance is probably of the order 3 to 1, whereas these two amino-sugars are present in the H substance in a ratio very close to 1. Differences in the quantitative composition of preparations of blood-group mucoids which show a single specificity, such as A or B or H, have been recorded in the literature, but it is obvious that significance cannot be attached to variations found in the amounts of a component sugar or amino-acid present in individual preparations unless it is known that these materials are essentially homogeneous on the basis of the results of physical, chemical, serological and immunochemical examination. There has been no opportunity so far of preparing from each of several cyst fluids specimens of group substance of a single specificity and established homogeneity and, until this has been accomplished, it would be inadvisable to express an opinion as to whether the recorded quantitative variations in the amounts of components do, in fact, establish that differences in composition occur in mucoids exhibiting similar blood-group specificity.

H substance isolated from cyst fluids by the methods described possesses no capacity to give a specific precipitate when mixed with pneumococcal

type XIV horse serum. In this respect, therefore, the H substance behaves as do the blood-group A and B substances and does not show the pneumococcal type XIV activity found to be associated with the Le^a substance prepared from cyst fluids under the same conditions. In our experience H substance isolated by a procedure which involves prolonged peptic digestion shows some reactivity with the pneumococcal type XIV antiserum.

Partial hydrolysis of the group substances with weak acetic acid causes them to react with pneumococcal type XIV horse antiserum and thus to develop an overlapping specificity with the specific polysaccharide of this organism. This pronounced cross-reactivity suggests that the *N*-acetylhexosamine-galactose chains which make up major structures within the polysaccharide moieties of the group substances are similar in pattern to each other and to the *N*-acetylglucosamine-galactose structures of the pneumococcus type XIV specific polysaccharide. According to Kabat, Baer, Bezer & Knaub (1948) and Kabat (1949), the fucose molecules in the undegraded group substances shield the *N*-acetylglucosamine-galactose structures common to these materials and prevent the cross-reactivity with the pneumococcal type XIV antiserum. The reactivity of the undegraded Le^a substance and the inactivity of the A and H substances with pneumococcal type XIV serum could perhaps be explained as a consequence of the higher glucosamine-chondrosamine ratio in the Le^a substance compared with the other group materials, with the result that the larger number of cross-reacting structures present cannot be adequately screened from the pneumococcal type XIV antibody by the fucose molecules, which presumably protrude from and in part dominate the essential surface configuration of the group substance.

Hydrolysis of the H substance with acetic acid rapidly liberates the whole of the fucose component, and it is of interest to record that to bring about a loss of half the original group specific activity shown by preparations of H, Le^a and A substances it is necessary to heat them with N-acetic acid for 15 min. 60 min. and about 150 min. respectively. The more rapid loss of group specific serological activity shown by the H substance is, therefore, probably correlated with the more ready liberation of fucose from the material.

The exact genetical relationship of the H substance described to the blood-group substances A and B is not yet clear. According to a theory proposed by Hirszfeld & Amzel (1940) and modified by Morgan & Watkins (1948) and by Boorman, Dodd & Gilbey (1948) the genes *A*, *B* and *O* are formed from a primary gene *H* by progressive mutations. Between the original gene form *H* and the completely mutated genes *A*, *B* and *O* there exists a series of transitional gene forms which give rise to A and H, B and H, and O and H substances on the surface of the A, B and O erythrocytes respectively. If a person possesses a single or double dose of Schiff's secretor gene, *S*, then the secretions will similarly contain A and H or B and H substances. As far as it is possible to determine at present there is no appreciable amount of a water-soluble substance which possesses group O character in the secretions of persons belonging to group O. The secretions from group O individuals are therefore found to possess H substance only. The hypothesis implies that when mutation is complete, gene forms designated A_c, B_c and O_c arise which are without an *H* gene component. It is in the serum of a person whose gene forms are devoid of H character that one would expect to find an anti-H agglutinin. This agglutinin, which is found very rarely, is neutralized in its action on group O cells by the human H substance. We have encountered two human sera which possessed useful anti-H agglutinins, one derived from a person belonging to group A and the second from a person belonging to group B. The cells of both individuals failed to react at room temperature with anti-H sera and therefore the erythrocytes seemed to be devoid of H substance as would be anticipated by the hypothesis. Evidence that the *H* gene was absent from the chromosome was sought by examining the donor's saliva for A and H, and B and H substances, respectively. Both persons were, however, found to be Lewis positive and non-secretors. Thus additional and essential evidence for establishing the absence of the *H* gene, by demonstrating the absence of a product of its activity, could not be obtained.

It is evident that if this hypothesis of the inheritance of the blood-group characters, based as it is on the activity of transitional gene forms, is correct then there are many far-reaching theoretical and practical consequences to consider, especially from the point of view of those engaged in the isolation and characterization of the blood-group substances. Perhaps the most important one at this stage in the development of the subject is to determine if the product of the activity of transitional genes—such as A_2 when present in homozygous form (A_2A_2)—is a single substance which possesses two distinct serological characters, A and H, or whether the products are two distinct substances each possessing a single serological specificity, A or H.

There remains the possibility that the H character is the result of the presence of a genetically independent blood-group system H–h. In the event of this being so it will be necessary to account for the undoubted influence of the *A*, *B* and *O* genes on the quantitative manifestation of the H character of erythrocytes, and on the amount of water-soluble H

substance in secretions. Predictions inherent in these hypotheses are open to a measure of experimental proof and attempts are now being made to obtain evidence for or against the ideas put forward.

SUMMARY

1. Methods for the isolation and purification of the human blood-group H substance from ovarian cyst fluids are described.

2. The H substance is a laevorotatory mucoid which contains about 5·3 % nitrogen and is similar in general composition and properties to the mucoids which are responsible for the human blood-group A, B and Lewis (Le^a) characters.

3. The H substance obtained is somewhat polydisperse, but is essentially homogeneous with respect to certain physical, chemical immunochemical and serological properties.

4. L-Fucose, D-galactose, D-glucosamine, D-chondrosamine and 11 amino-acids have been identified as components of the H substance.

5. The acid hydrolysis products contain 14 % fucose, 54 % reducing substances, expressed as glucose, and 31 % hexosamine. The α-amino-acid nitrogen is equivalent to about 41 %, and the α-amino nitrogen to 88 % of the total nitrogen.

6. Possible relationships between the products of the *A*, *B* and *O* genes and the H substance are discussed.

The authors thank the University of London for a grant to purchase the Uvispek spectrophotometer used and one of us (E.F.A.) is indebted to the Medical Research Council for a Studentship.

REFERENCES

Aminoff, D. & Morgan, W. T. J. (1948). *Nature, Lond.*, **162**, 579.
Aminoff, D. & Morgan, W. T. J. (1951). *Biochem. J.* **48**, 74.
Aminoff, D., Morgan, W. T. J. & Watkins, W. M. (1950). *Biochem. J.* **46**, 426.
Aminoff, D., Morgan, W. T. J. & Watkins, W. M. (1952). *Biochem. J.* **51**, 379.
Annison, E. F., James, A. T. & Morgan, W. T. J. (1951). *Biochem. J.* **48**, 477.
Annison, E. F. & Morgan, W. T. J. (1950). *Nature, Lond.*, **165**, 884.
Annison, E. F. & Morgan, W. T. J. (1952). *Biochem. J.* **50**, 460.
Bendich, A., Kabat, E. A. & Bezer, A. E. (1946). *J. exp. Med.* **83**, 485.
Boorman, K., Dodd, B. & Gilbey, B. E. (1948). *Ann. Eugen., Camb.*, **14**, 201.
Burnet, F. M., McCrea, J. F. & Anderson, S. G. (1947). *Nature, Lond.*, **160**, 404.
Donleavy, J. (1936). *J. Amer. chem. Soc.* **58**, 1004.
Grubb, R. (1951). *Acta path. microbiol. scand.* **28**, 61.
Grubb, R. & Morgan, W. T. J. (1949). *Brit. J. exp. Path.* **30**, 198.
Hallauer, C. (1934). *Z. ImmunForsch.* **83**, 114.
Hirszfeld, L. & Amzel, R. (1940). *Ann. Inst. Pasteur*, **65**, 251.
Hough, L., Jones, J. K. N. & Wadman, W. H. (1949). *J. chem. Soc.* p. 2511.
Johns, R. G. S. & Marrack, J. R. (1952). *Biochem. J.* **50**, xvii.
Kabat, E. A. (1949). *Bact. Rev.* **13**, 189.
Kabat, E. A., Baer, A., Bezer, A. E. & Knaub, V. (1948). *J. exp. Med.* **87**, 295.
Kabat, E. A., Baer, A. & Knaub, V. (1949). *J. exp. Med.* **89**, 1.
Kabat, E. A., Bendich, A., Bezer, A. E. & Beiser, S. (1947). *J. exp. Med.* **85**, 685.
Kekwick, R. A. (1950). *Biochem. J.* **46**, 438.
Kekwick, R. A. (1952). *Biochem. J.* **50**, 471.
King, H. K. & Morgan, W. T. J. (1944). *Biochem. J.* **28**, x.
Landsteiner, K. & Harte, R. A. (1941). *J. biol. Chem.* **140**, 673.
Morgan, W. T. J. (1943). *Brit. J. exp. Path.* **24**, 41.
Morgan, W. T. J. (1946). *Biochem. J.* **40**, xv.
Morgan, W. T. J. & Elson, L. A. (1934). *Biochem. J.* **28**, 988.
Morgan, W. T. J. & Van Heyningen, R. (1944). *Brit. J. exp. Path.* **25**, 5.
Morgan, W. T. J. & Waddell, M. B. R. (1945). *Brit. J. exp. Path.* **26**, 387.
Morgan, W. T. J. & Watkins, W. M. (1948). *Brit. J. exp. Path.* **29**, 159.
Muther, A. T., Tollens, B. (1904). *Ber. dtsch. chem. Ges.* **37**, 308.
Schiff, F. & Sasaki, H. (1932). *Z. ImmunForsch.* **77**, 129.
Sevag, M. G. (1934). *Biochem. Z.* **273**, 419.
Stepanov, A., Kuzin, A., Markageva, Z. & Kosyakov, P. (1940). *Biochemistry, Lenigr.*, **5**, 547.
Witebsky, E. & Klendshoj, N. C. (1941). *J. exp. Med.* **73**, 655.
Wolfrom, M. L. & Christman, C. C. (1931). *J. Amer. chem. Soc.* **53**, 3413.

ADDENDUM

Physicochemical Examination of Blood-Group H Substance

By R. A. KEKWICK
The Lister Institute, London, S.W. 1

(*Received* 31 *January* 1952)

The H substance was examined in the electrophoresis apparatus and ultracentrifuge, and in addition the partial specific volume and diffusion constant were determined.

METHODS

Electrophoresis. The material was examined in a Tiselius electrophoresis apparatus at 0°, using the diagonal schlieren optical system and monochromatic light, $\lambda = 546$ mμ.,

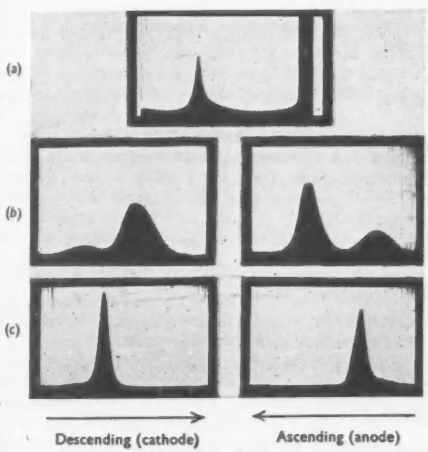


Fig. 1. Sedimentation diagram and electrophoresis patterns of blood-group H substance. (*a*) Sedimentation after 60 min. at 270 000 *g*. 1% (w/v) in phosphate-NaCl. (Phosphate, pH 8, $I = 0{\cdot}2$; NaCl, 0·15 M.) (*b*) Electrophoresis pattern after 180 min. at 5 V./cm. of 2% (w/v) solution in phosphate, pH 8, $I = 0{\cdot}2$. (*c*) Electrophoresis pattern after 220 min. at 5 V./cm. of 2% (w/v) solution in acetate, pH 4, $I = 0{\cdot}1$. Lower arrows show direction of migration in both (*b*) and (*c*).

isolated from a mercury arc by a suitable filter. As the main object of this examination was to detect any traces of contaminating substances of high molecular weight, solutions of salt-free material were made up in suitable buffers to give a concentration of 2 g./100 ml., and dialysed against buffer overnight.

Sedimentation. The ultracentrifugal examination was carried out in a Svedberg oil turbine machine at 60 000 rev./min. (270 000 *g*) using an optical system similar to that of the electrophoresis apparatus. The variation of sedimentation constant with concentration was examined over a limited concentration range. Solutions were made up in suitable buffers and sufficient NaCl added to suppress any charge effect on sedimentation.

Diffusion. The diffusion measurements were made by Dr A. G. Ogston, using the method of Coulson, Cox, Ogston & Philpot (1948). Values for the diffusion constant and sedimentation constant were corrected to water at 20° (Svedberg & Pedersen, 1940).

Partial specific volume. This was determined pyknometrically at 25°, the substance being dissolved in the buffer used for the majority of the sedimentation constant measurements.

RESULTS

In acetate buffer, pH 4, $I = 0{\cdot}1$, after prolonged electrophoresis only one component was demonstrable. At pH 4 the boundaries were symmetrical and showed very little spreading. At pH 8 the ascending boundary was symmetrical though the apex of the peak exhibited a slight flattening, and the descending boundary was asymmetrical (Fig. 1). The spreading of both boundaries at pH 8 was considerably greater than at pH 4. Migration in each instance was anodic, but very slight at pH 4.

Table 1. *Sedimentation and diffusion constants of* H *substance*

(Sedimentation and diffusion constants corrected to water at 20° (Svedberg & Pedersen, 1940).)

Concentration (g./100 ml.)	$S_{20} \times 10^{13}$ (corr.)	$D_{20} \times 10^{7}$ (corr.)
0·50	6·64	—
0·75	6·04	—
1·00	5·21	1·21

In the ultracentrifuge at pH 8 only one component was present, and the peak remained relatively sharp even after 90 min. at 270 000 *g* in a 1% (w/v) solution, and at 0·5% (w/v) a satisfactory peak was present after 60 min. at 270 000 *g*. The sedimentation constant was determined at three concentrations and the data of Table 1 show that there is a marked variation of sedimentation constant with concentration.

Included in Table 1 is a value for the diffusion constant kindly measured by Dr A. G. Ogston. The partial specific volume of the substance was 0·636.

From the sedimentation and diffusion constant values obtained in a solution containing 1 g./100 ml. H substance, and using the determined value of the partial specific volume, the molecular weight was calculated from the usual equation (Svedberg & Pedersen, 1940) giving a value of 320 000. From the same data the frictional ratio f/f_o obtained was 4·2. This corresponds to an axial ratio of >100, or may be interpreted as indicating a high degree of hydration.

DISCUSSION

The data indicate that electrophoretically the substance is essentially homogeneous, which means that the nitrogen in the preparation is not attributable to a protein contaminant but is an integral part of the molecule.

For a substance of molecular size 320 000 associated with the observed large frictional ratio which is reflected in the concentration dependence of the sedimentation constant, a rather sharper boundary would be expected to be maintained throughout sedimentation if the substance was strictly monodisperse. The molecular size appears to be rather higher than that previously reported for blood-group A substance (Kekwick, 1950) and Le^a substance (Kekwick, 1952), and there is evidence of a higher degree of molecular asymmetry.

The conclusion must probably be drawn that the substance is moderately polydisperse, but the data are insufficient to give a precise expression of the range and distribution of molecular size in the preparation.

SUMMARY

1. The blood-group H substance has been found essentially homogeneous electrophoretically.

2. The mean molecular weight of the substance calculated from sedimentation and diffusion data is 320 000 and the frictional ratio 4·2.

3. The substance is probably moderately polydisperse and the molecular shape highly asymmetrical.

I wish to acknowledge the technical assistance of Mr H. Murray.

REFERENCES

Coulson, C. A., Cox, J. T., Ogston, A. G. & Philpot, J. St L. (1948). *Proc. roy. Soc.* A, **192**, 382.
Kekwick, R. A. (1950). *Biochem. J.* **46**, 438.
Kekwick, R. A. (1952). *Biochem. J.* **50**, 471.
Svedberg, T. & Pedersen, K. O. (1940). *The Ultracentrifuge.* Oxford University Press.

Citric Acid and Bone Metabolism

By T. F. DIXON and H. R. PERKINS
Institute of Orthopaedics, London, W. 1

(*Received* 18 *February* 1952)

Since Dickens (1941) showed that 90% or more of the citric acid of the body was located in the skeleton, and Class & Smith (1943) demonstrated its presence in various body concretions, evidence has been accumulating that this substance plays an important part in the metabolism of bone. A connexion between calcium and citric acid was established when Pincus, Peterson & Kramer (1926) found that these substances formed a soluble complex which is assumed to account for the non-ionizable part of the diffusible fraction of serum calcium. Gomori & Gulyas (1944) showed that citrate injections in dogs made serum calcium more ultrafiltrable so that a rapid urinary excretion of calcium took place, and similar results were obtained in dogs by Chang & Freeman (1950). A rise in serum calcium caused by parathormone was shown by Alwall (1944) to be accompanied by a rise in serum citrate, and in Gomori's experiments, in which dogs were injected with citrate, microscopic examination of the bone showed a picture similar to that observed after toxic doses of parathormone. The connexion between serum calcium and citrate was further established by Freeman & Chang (1950). These authors found that high doses of vitamin D administered to dogs raised not only the serum calcium but also the serum citrate, and suggested that this was due to increased liberation of these substances from the bone reservoir. A possible explanation for the presence of citrate in bone was indicated by the work of Kuyper (1938), who showed that the precipitate of calcium, phosphate and citrate formed *in vitro* in the presence of suitable concentrations of these substances is actually a complex such as may exist in bone. He also found that the solubility of the precipitate was increased

by the presence of citrate and magnesium in the solution.

The facts make it seem likely that calcification mechanisms may be influenced by the amount of citrate present in the circulating fluids and at the site of calcification. A study in bone of the enzyme systems concerned with the well known citric acid cycle seemed of interest in elucidating the role of citric acid in this tissue. Thus the enzymes concerned with the formation and further conversion of citric acid, namely citrogenase, aconitase and *iso*citric dehydrogenase, were studied in different bone regions. The effect of citric acid on the *in vitro* calcification of tibia slices from rachitic rats was also investigated.

EXPERIMENTAL

Calcification of bone slices from rachitic rats. The young white rats used were maintained on the 'Glaxo' rachitogenic diet for 14 days and then sacrificed. Immediately after death the tibiae were dissected out and scraped free of adhering tissue. Thin sagittal sections were then cut and incubated in the solution containing calcium and phosphate, with or without the addition of Mg or citrate. The basal solution contained NaCl, 0·075 M; $NaHCO_3$, 0·022 M; KCl, 0·005 M; $CaCl_2$, 0·002 M; sodium phosphate buffer, 0·0017 M, pH 7·4. This gives concentrations of Ca = 8 mg./100 ml. and P = 5 mg./100 ml. After incubation at 37° for the desired period, the slices were removed, washed in distilled water, and then stained with 1% (w/v) $AgNO_3$, washed, and allowed to blacken under the influence of light. With each pair of experimental sections two control sections were run, one incubated in the basal medium alone, and one in the same solution without Ca or P.

Preparation of tissues for enzyme experiments. For aconitase estimation the tissues were ground in a mortar with washed silver sand and 10 ml./g. of 0·05 M-phosphate, pH 7·4, and the centrifuged extract was used for determination of the enzyme content. In agreement with the results of Johnson (1939), the aqueous extract was found to contain practically all the activity of the whole tissue. Although there was a possibility that phosphate might, by its affinity for calcium, tend to prevent extraction of the enzyme from the bone, phosphate buffer and 0·9% NaCl solution were, in fact, found to extract the same amount of aconitase. It was convenient to use extracts, since they contained only small amounts of citric acid compared with the whole bone, and thus did not tend to obscure the enzymic formation from *cis*-aconitate.

When citrogenase or *iso*citric dehydrogenase were being estimated, the kidney and liver were finely minced, and the bone was chopped as uniformly and finely as possible. *iso*-Citric dehydrogenase was demonstrated qualitatively by the use of sliced bone, but attempts to show the enzyme in bone brei or extracts were completely unsuccessful. It was therefore concluded that excessive destruction of the tissue led to breakdown or inactivation of the small quantities of enzyme present in bone. For this reason, in subsequent quantitative experiments, chopped bone was employed, since the physical properties of the rabbit bones used prevented the cutting of slices. Experiments on the optimal conditions for the demonstration of the citrogenase content of the tissues also led to the conclusion that the minimum of destruction gave the best results. For example, in an experiment with rat liver, the following results were obtained: mg. citric acid formed/g. tissue (*a*) finely minced, 7·5; (*b*) whole homogenate, 5·0; (*c*) supernatant after centrifugation of homogenate, 0·7; (*d*) residue resuspended, 1·1. Minced tissue was used rather than slices so that a comparison could be made with the activity of bone.

Rabbit tissues were obtained from animals weighing about 600–700 g. The tissues studied were the kidneys, liver, red marrow, newly calcified metaphysis with the bony trabeculae, epiphyseal line cartilage and diaphyseal cortex of the tibiae and femora. After dissection, histological examination of the parts used showed that the portion described as metaphysis consisted mainly of bony trabeculae, which showed some cartilage inclusions, and a slight contamination with the cartilage of the epiphyseal plate. The epiphyseal cartilage portion consisted of the remainder of the epiphyseal plate, with a few bony trabeculae attached to the part opposite the metaphysis. The marrow and cortex specimens were uncontaminated by other tissue.

Estimation of citrogenase activity of tissues. The method used for estimation of the enzyme system which forms citrate from oxaloacetate and acetate was based upon the technique of Stern & Ochoa (1951). These authors point out that the reaction involves the conversion of acetate to acetyl phosphate, the transfer of the acetyl group to form acetyl-coenzyme A, and the final condensation of the acetyl group with oxaloacetic acid to form citric acid. In the present investigation the system as a whole was studied, and is referred to for convenience as 'citrogenase'.

The animals were killed by stunning and bleeding, and samples of 0·100 g. of finely minced kidney, liver and marrow, and about the same weight of chopped bone, were used for the experiments. The incubation medium consisted of 2 ml. of a solution containing sodium acetate, 0·01 M; KCl, 0·1 M; $MgSO_4$, 0·003 M; phosphate, pH 7·4, 0·005 M; cysteine, 0·01 M; adenosinetriphosphate (ATP), 0·002 M; oxaloacetate, 0·01 M. The final pH of the reaction mixture was brought to 7·4. In the control experiments the oxaloacetate was replaced by 0·01 M-acetate, so that when bone was used extraction of the tissue citrate during the incubation took place to the same extent in the control as in the experimental tubes. The weighed tissue was added to the medium and incubated at 28° for 1 hr. with constant agitation. It was shown experimentally that even with an active tissue like kidney the rate of formation was essentially constant during the first hour. The reaction was stopped by the addition of 1 ml. of 25% (w/v) trichloroacetic acid (TCA), and the supernatant after centrifugation was used for citrate determination. In the case of the bone, in order to demonstrate the formation of relatively small amounts of citrate in the presence of the large amounts contained in the tissue, after incubation the tubes were cooled in ice and centrifuged. The supernatant, together with water with which the residue had been further washed, was then treated with TCA as above. Duplicate experiments showed that this method gave a sufficiently uniform and small extraction of the bone citrate to enable the formation of citric acid in the supernatant by the enzyme reaction to be estimated. It was also found that when bone was first boiled to inactivate its enzymes, no citrate formation was observed by this method. In view of the low bone citrogenase activity observed, it was thought possible that the system might be lacking in coenzyme A (see Stern & Ochoa, 1951). Therefore, in one series of experiments, coenzyme A powder prepared from

pigeon liver, and shown by activation of sulphanilamide acetylation to have an activity of 1·4 units/mg. (Kaplan & Lipmann, 1948), was added to the medium. However, little if any increased citrate formation was observed and therefore coenzyme A was not employed in subsequent experiments.

Estimation of aconitase activity of tissues. The method of estimating aconitase activity was based upon that of Johnson (1939). *cis*-Aconitic anhydride was prepared by the method of Anschütz & Bertram (1904) and neutralized with $NaHCO_3$ in the cold immediately before use. The sample thus prepared contained no citric acid. For aconitase estimation, 0·5 ml. of the centrifuged tissue extract was incubated at 37° with 0·5 ml. of a solution containing the equivalent of 1 mg. of *cis*-aconitic anhydride. The period chosen was such that not more than 50% of the *cis*-aconitate had been converted to citrate, in the case of the bone, 1 hr., and for the soft tissues, 10 min. After incubation, the reaction was stopped by the addition of 0·5 ml. of 25% (w/v) TCA and the resulting solution was used for citric acid determination.

Estimation of isocitric *dehydrogenase activity of tissues.* Our first successful qualitative demonstrations of *iso*citric dehydrogenase activity in bone were carried out according to the technique of Follis & Berthrong (1949). Thin free-hand sections of freshly dissected rat bone (humerus was found to be the most suitable because of the strong trabeculae in the epiphysis) were cut to include the epiphysis, epiphyseal line and the metaphysis. The sections were incubated anaerobically on a microscope slide at 37° with 0·1 M substrates dissolved in physiological saline solution (Robinson, 1949). Dehydrogenase activity was observed in a low-power microscope by the reduction of either methylene blue, with which the sections had been previously treated, or triphenyltetrazolium chloride contained in the solution (1 mg./ml.), the latter forming a red insoluble compound on reduction.

Triphenyltetrazolium was then used for the quantitative determination of the enzyme according to the method of Kun & Abood (1949). Although the substrate for the enzyme is *iso*citric acid, the substance added to the medium in these experiments was citric acid. Since the tissues have been shown in other experiments (see below) to contain sufficient aconitase to ensure the establishment of the equilibrium

$$\text{citrate} \rightleftharpoons \textit{cis}\text{-aconitate} \rightleftharpoons \textit{iso}\text{citrate},$$

this procedure was considered to be justified. To the weighed tissue (about 0·1 g.) were added 1·5 ml. of 0·05 M-phosphate, pH 7·4, 0·5 ml. of water or 0·2 M-sodium citrate and 0·5 ml. of 0·2% (w/v) triphenyltetrazolium chloride. After mincing, the tubes were incubated at 37° with intermittent shaking for a suitable period, which was found to be 10 min. for the kidney and liver, and 2 hr. for the bone. After incubation the tubes were cooled in ice and 7 ml. of acetone were added to each and shaken. The mixture was centrifuged and the supernatant, after filtration if necessary, was used for comparison of colour intensity against an arbitrary standard. The amount of reduced dye present was then read from a standard curve prepared from known amounts of triphenyltetrazolium, reduced under standard conditions by $Na_2S_2O_4 . 2H_2O$. By this method, a reproducible straight-line relationship between colour intensity and concentration was obtained.

Oxidative removal of citrate by minced tissues. In the experiments on the oxidative removal of added citrate the following technique was employed. To weighed samples of minced kidney or chopped bone 7·2 ml./g. of a physiological salt solution, pH 7·4 (Krebs & Henseleit, 1932), containing 7 mg./ml. of citric acid, were added. The gaseous phase used was 5% (v/v) CO_2 in O_2 at atmospheric pressure, and the flasks were incubated at 37° with constant agitation for the desired period. The reaction was stopped by the addition of 1 ml. of 25% (w/v) TCA, and after centrifugation the supernatant was used for citric acid determination. In the bone experiments the same technique of centrifugation before the addition of TCA was used as that described in the method for citrogenase.

Estimation of the alkaline phosphatase content of tissues. The tissue was treated with 20 ml./g. of $CHCl_3$-saturated water, ground with washed silver sand and allowed to autolyse overnight at 3°. The centrifuged extract was used for alkaline phosphatase determination by a method essentially similar to that of Shinowara, Jones & Reinhart (1942).

Determination of citric acid. Citric acid was determined in the TCA extracts by the colorimetric method of Weil-Malherbe & Bone (1949). A standard curve was employed, and a standard was included with each determination.

RESULTS

Influence of citrate on the calcification of rachitic cartilage. Following the technique of Robison (1932) the influence of magnesium and citrate on the *in vitro* calcification of bone slices from rachitic rats was studied. The concentrations used were only a little higher than those normally occurring in blood. A number of experiments was performed, and typical results are given in Table 1. From these it can be seen that, although magnesium (as sulphate) at a level of 1×10^{-3} M produced only a slight inhibitory effect on calcification, sodium citrate at 5×10^{-4} M produced a marked inhibition. This amount of citrate, which is slightly greater than the normal level in blood, would be sufficient to form a complex with about 3 mg./100 ml. of the ionized calcium. Shipley, Kramer & Howland (1926) had previously shown that calcification is inhibited if calcium is supplied as calcium citrate. Here it is shown that the addition of citrate equivalent to a part only of the calcium has the same effect.

Table 1. *Inhibition of* in vitro *calcification of bone slices from rachitic rats by citrate and magnesium*

(Degree of calcification was graded from 0 (no calcification) to + + + + (complete calcification).)

	Calcification after	
	8 hr.	17 hr.
Basal medium	+ +	+ + + +
Basal medium + 5×10^{-4} M-citrate	0	+
Basal medium + 5×10^{-4} M-citrate + 1×10^{-3} M-magnesium	0	+
Basal medium + 1×10^{-3} M-magnesium	+ +	+ + +

Citrogenase. Rat or rabbit kidney and liver were found to form citrate very readily under the conditions used, and, in one experiment with rat kidney, 32% of the oxaloacetate was converted into citrate, a figure higher than those usually reported in the literature (e.g. Green, Loomis & Auerbach (1948), 18%). Much lower citrogenase activities were found in bone, and the comparison between these results and those for kidney and liver is given below.

Table 2. *Aconitase activities of rib parts of young rats*

Part of rib	Aconitase activity (mg. citric acid formed/g. tissue/hr.)
Bone	10·2
Costochondral junction	18·3
Cartilage	9·4

Aconitase. Of the three enzymes of the citric acid cycle studied, aconitase was found in greatest amount in bone. In addition to the results obtained for kidney, liver and various parts of the bones of rabbits given below, it is of interest to mention that the ribs of young rats, and the costochondral junctions in particular, were also shown to have a high aconitase activity (Table 2). Here, as with the rabbit bone, the actively calcifying region had a higher enzyme activity than either the bony or cartilaginous rib.

iso*Citric dehydrogenase.* Bone slices produced considerable reduction of hydrogen-accepting dye when either succinate or citrate was present as a substrate. Typical results obtained with the humerus of a young rat are given in Table 3. The region in which reduction of dye first appeared was that in the immediate vicinity of the centre of the epiphyseal-line cartilage. As the experiment proceeded the area of reduction extended to include a roughly circular area centred on the region where a change was first observed. The reduced form of triphenyltetrazolium chloride did not reoxidize in air as did that of methylene blue, and the former dye was therefore used in the quantitative experiments.

Comparison of activities of citrogenase, aconitase, isocitric dehydrogenase, alkaline phosphatase and of content of citric acid in rabbit tissue. Enzyme activities and citric acid contents of the various tissues are given in Table 4. Alkaline phosphatase is included for comparison. Citric acid contents of the kidney, liver and marrow were very low indeed, whereas considerable amounts were found in each of the bone regions studied, hard fully calcified cortex having the highest content. The citrogenase, aconitase, *iso*citric dehydrogenase and phosphatase activities of the metaphysis and epiphyseal cartilage are much greater than those of the cortex. In order to facilitate comparison, the enzyme activity of the more active parts of the bone are given in Table 5 as fractions of the activity of the kidney and liver respectively. From this it can be seen that the citrogenase activities of the metaphysis and epiphyseal plate are about one-quarter and one-sixth respectively of the activity of the kidney, whereas the

Table 3. *Dehydrogenase activities of rat humerus slices*

(Dye reduction observed under a low-power microscope. Dehydrogenase activities graded from 0 (no activity) to + + + + (maximum activity).)

Added substrate	Condition	Reduction of dye: Methylene blue	Reduction of dye: Triphenyltetrazolium
None	Aerobic	0	+
None	Anaerobic	+	+
Acetate	Anaerobic	+	+
Citrate	Anaerobic	+ + + +	+ + + +
Succinate	Anaerobic	+ + +	+ + +

Table 4. *Citric acid content and enzymic activities of rabbit tissues*

(Citrogenase and aconitase activities expressed as mg. citric acid produced/g. tissue/hr. *iso*Citric dehydrogenase activity expressed as mg. *iso*citric acid decomposed/g. tissue/hr. calculated from dye reduction. Alkaline phosphatase activity expressed as mg. P liberated/g. tissue/hr. Number of determinations given in brackets after each figure.)

Tissue	Citrogenase	Aconitase	*iso*Citric dehydrogenase	Alkaline phosphatase	Citric acid (mg./g.)
Kidney	2·12 (4)	133 (3)	2·05 (4)	3·78 (2)	—
Liver	1·27 (4)	54 (3)	1·37 (4)	0·62 (2)	—
Marrow	0·26 (4)	8·6 (2)	0·014 (3)	0·99 (2)	—
Metaphysis	0·51 (3)	11·9 (2)	0·041 (4)	5·49 (2)	6·7 (4)
Epiphyseal line cartilage	0·31 (4)	12·1 (3)	0·038 (3)	3·15 (2)	7·1 (4)
Bone cortex	0·04 (3)	0·61 (3)	0 (4)	0·41 (2)	9·5 (4)

Table 5. *Ratios of enzyme activities in rabbit bone in kidney and liver*

(Number of determinations given in brackets after each figure.)

Ratio	Citrogenase	Aconitase	*iso*Citric dehydrogenase
Metaphysis/kidney	0·25 (3)	0·096 (2)	0·023 (4)
Epiphyseal line cartilage/kidney	0·16 (4)	0·096 (2)	0·017 (3)
Metaphysis/liver	0·45 (3)	0·222 (2)	0·043 (4)
Epiphyseal line cartilage/liver	0·28 (4)	0·210 (2)	0·025 (3)

*iso*citric dehydrogenase activities of the same tissues are only about one-fortieth and one-sixtieth respectively of the kidney value. The ratios for the aconitase lie somewhere between. It thus appears that in the bony tissues there is a relative excess of the citric acid-forming enzyme over the citric acid-removing enzyme, since the aconitase merely serves to establish the equilibrium

citrate (89·5 %) ⇌ aconitate (4·3 %) ⇌ *iso*citrate (6·2 %)

(Krebs & Eggleston, 1943).

Oxidative removal of citrate. The very low level of *iso*citric dehydrogenase present in bone was confirmed in experiments on the removal of added citrate. Whereas the kidney showed considerable activity by the method used (disappearance of added citrate after 1 hr., 29 %; after 2 hr., 43 %; after 4 hr., 61 %) no continued removal by bone could be demonstrated (disappearance of added citrate: after 1 hr., 10 %; after 2 hr., 10 %; after 4 hr., 10 %). The small initial disappearance of citrate was evidently due to the activity of the aconitase present, which converted a small proportion of the citrate into aconitate and *iso*citrate. This hypothesis was confirmed by use of a kidney extract containing aconitase but no dehydrogenase, which had the same effect of causing the disappearance of a constant small fraction of the added citrate.

Table 6. *Removal by minced rat kidney of citrate added either as bone powder or as sodium citrate*

(Approx. 7 mg. citric acid added per g. kidney in each case.)

Exp. no.	Form of added citrate	Incubation (hr.)	Citrate found (% of initial)
1	Acetone-dried rat bone powder	0	100
		1	87
		3	81
	Sodium citrate	0	100
		1	39
		3	12
2	Ox-bone cortex powder (300 mesh)	0	100
		1	97
		3	96
	Sodium citrate	0	100
		1	58
		3	14

The availability of bone citrate for enzymic removal. The active aconitase and *iso*citric dehydrogenase of rat kidney were used in an attempt to metabolize further the citrate present in bone. The bone used was either a fine powder made from acetone-dried rat bone, or powdered ox-bone cortex (300-mesh). The activity of the minced kidney used was shown by a parallel experiment in which citrate was added in the form of sodium citrate. The results are given in Table 6, from which it can be seen that the citrate present in the rat bone was removed to a small extent by the active kidney enzymes, whilst in the more highly calcified ox-bone cortex only a slight amount of the citrate was removed.

DISCUSSION

It appears that all the parts of bone examined have, by comparison with other tissues, e.g. kidney or liver, citrogenase and aconitase activities much greater than those of *iso*citric dehydrogenase. The mechanism for the production of a local high concentration of citric acid therefore exists in bone. Such citric acid may be co-precipitated with calcium phosphate during its deposition, in which case it may influence the amount of deposition by holding up calcium in a complex form. A high citrate concentration could even reverse the process of calcification and solubilize bone salt already laid down. Excess of citrogenase over *iso*citric dehydrogenase in marrow does not lead to citrate deposition since no bone salt is laid down anyway.

SUMMARY

1. It has been shown that the 'citrogenase' and aconitase activities of various rabbit bone regions are relatively greater than that of *iso*citric dehydrogenase compared with kidney and liver.

2. In extension of earlier observations increasing concentrations of citrate inhibit the calcification of hypertrophic rat cartilage *in vitro*.

3. Enzyme levels in bone indicate the relatively greater metabolic activities of metaphysis and epiphysis over that of cortex.

4. Amounts of citrate in bone regions are in inverse ratio to their metabolic activities.

5. It is suggested that the higher 'citrogenase' and lower *iso*citric dehydrogenase levels in bone regions may produce local increased concentrations of citric acid which may then become co-precipitated during deposition of bone salt.

The authors are grateful to Dr D. Herbert for the gift of some oxaloacetic acid.

REFERENCES

Alwall, N. (1944). *Acta med. scand.* **116**, 337.
Anschütz, R. & Bertram, W. (1904). *Ber. dtsch. chem. Ges.* **37**, 3967.
Chang, T. S. & Freeman, S. (1950). *Amer. J. Physiol.* **160**, 330.
Class, R. N. & Smith, A. H. (1943). *J. biol. Chem.* **151**, 363.
Dickens, F. (1941). *Biochem. J.* **35**, 1011.
Follis, R. H. & Berthrong, M. (1949). *Bull. Johns Hopk. Hosp.* **85**, 281.
Freeman, S. & Chang, T. S. (1950). *Amer. J. Physiol.* **160**, 341.
Gomori, G. & Gulyas, E. (1944). *Proc. Soc. exp. Biol., N.Y.*, **56**, 226.
Green, D. E., Loomis, W. F. & Auerbach, V. H. (1948). *J. biol. Chem.* **172**, 389.
Johnson, W. A. (1939). *Biochem. J.* **33**, 1046.
Kaplan, N. O. & Lipmann, F. (1948). *J. biol. Chem.* **174**, 37.
Krebs, M. A. & Eggleston, L. V. (1943). *Biochem. J.* **37**, 334.
Krebs, H. A. & Henseleit, K. (1932). *Hoppe-Seyl. Z.* **210**, 33.
Kun, E. & Abood, L. G. (1949). *Science*, **109**, 144.
Kuyper, A. C. (1938). *J. biol. Chem.* **123**, 405.
Pincus, J. B., Peterson, H. A. & Kramer, B. (1926). *J. biol. Chem.* **68**, 601.
Robinson, J. R. (1949). *Biochem. J.* **45**, 68.
Robison, R. (1932). *The Significance of Phosphoric Esters in Metabolism.* New York: University Press.
Shinowara, G. Y., Jones, L. M. & Reinhart, H. L. (1942). *J. biol. Chem.* **142**, 921.
Shipley, P. G., Kramer, B. & Howland, J. (1926). *Biochem. J.* **20**, 379.
Stern, J. R. & Ochoa, S. (1951). *J. biol. Chem.* **191**, 161.
Weil-Malherbe, H. & Bone, A. D. (1949). *Biochem. J.* **45**, 377.

The Determination of Glucosamine by Alkaline Decomposition

By M. V. TRACEY
Rothamsted Experimental Station, Harpenden, Hertfordshire

(*Received* 12 *March* 1952)

Estimation of free glucosamine is usually done by the method of Elson & Morgan (1933) or by modifications of this method. Some workers have found difficulty in getting reproducible results unless conditions are most rigorously standardized (Ogston & Stanier, 1950; Johnston, Ogston & Stanier, 1951; Schloss, 1951). This may be explicable on the grounds that both the initial reactants, the amino sugar and acetylacetone, are unstable under the conditions of reaction and that several products are formed, including a number of chromogens (Schloss, 1951). The specificity of the method is not complete; acetylglucosamine gives a similar colour and so do mixtures of amino-acids and sugars (Sideris, Young & Krauss, 1938; Horowitz, Ikawa & Fling, 1950; Immers & Vasseur, 1950). Urea and glyceraldehyde have also been reported as interfering (Lutwak-Mann, 1941). Recently a new colorimetric method in which deamination of the amino sugar precedes its estimation by heating with indole and hydrochloric acid has been proposed by Dische & Borenfreund (1950). This has been reported as giving high results in the presence of other sugars (McCrea, 1951). It has been found that an essentially similar method which had been developed using orcinol for colour development after deamination also gave high results in the presence of other sugars.

The method presented here which has been communicated in a preliminary form (Tracey, 1951) is based on the observation by Morgan (1936) that glucosamine and galactosamine 'yield practically the whole of their nitrogen in the form of ammonia on heating for a few minutes with normal alkali at 100°'. Conditions under which the yield of ammonia is quantitative and in which interference by other substances is minimal are described.

EXPERIMENTAL

Reagents

Phosphate-borate mixture. Saturated Na_3PO_4 is saturated with $Na_2B_4O_7.10H_2O$ at room temperature (at 14°, 1 l. of saturated Na_3PO_4 will dissolve about 42 g. of borate).

Nessler's reagent. This was prepared after Vanselow (1940). 45·5 g. HgI_2 and 34·9 g. KI are dissolved in as little water as possible, 112 g. KOH added and the solution diluted to 1 l.

Procedure

The sample, which should be 2 ml. or less in volume and not strongly acid, is introduced into a Markham (1942) still. The sample is washed in with a total of 5 ml. of the phosphate-borate mixture. Distillation is then carried out slowly (about 3 ml. distillate/min.), the distillate being collected in a test tube matched for use in a photoelectric colorimeter and graduated at 10 ml. Before use 1 ml. of 2% (w/v) boric acid is placed in each tube. Distillation is stopped when the distillate reaches the 10 ml. mark on the receiving tube. Blanks are distilled at the beginning and end of a set of distillations, and also solutions of $(NH_4)_2SO_4$

of suitable concentration to act as standards. All tubes of distillate are brought to the same temperature by standing in a vessel of water, and 1 ml. of Nessler's reagent added to each. After mixing, the tubes are allowed to stand 30 min. and are read in a photoelectric colorimeter, using a filter (Ilford 623) with maximum transmission in the region of 490 mμ. With $(NH_4)_2SO_4$ standards a straight line relation between the instrument reading and the amount of NH_3 distilled is obtained over a range of 5–40 μg. N (60–500 μg. amino sugar). Alternatively, the NH_3 may be titrated with dilute HCl in the presence of a suitable indicator (Conway & O'Malley, 1942) using the photoelectric colorimeter to get an exact and reproducible end point.

The sensitivity may be increased either by using a colorimeter in which the light path through the nesslerized distillate is longer (an increase by a factor of 2·5 was obtained by using a 4 cm. cell) or by reducing the volume of distillate collected. Quantitative recoveries of NH_3 were achieved from glucosamine, collecting only 4 ml. of distillate, by preheating the phosphate-borate glucosamine mixture before distillation. Steam was allowed to pass through the still jacket for 3 min. before beginning a slow distillation.

Choice of alkali. Yields of NH_3 on distillation of glucosamine with 0·5 N-Na_2CO_3, saturated Na_2CO_3, and saturated $Na_2B_4O_7$ were about 50, 80 and 25%. Borate buffers (approx. 0·2 M) of pH 10·0, 11·1 and 12·2 gave yields of 20–30%. The phosphate-borate-NaOH mixture of Pucher, Vickery & Leavenworth (1935) gave results similar to those of borate buffers. Saturated Na_3PO_4 (pH approx. 11·4) gave theoretical yields of NH_3. When glucose is distilled with saturated Na_3PO_4 volatile products are formed that give a colour with Nessler's reagent and a cloudiness that increases with time. Saturation of the phosphate solution with borate stops this interference while causing little change in the pH of the solution and allowing quantitative NH_3 recoveries from glucosamine and galactosamine. Quantitative recoveries are also obtained from biosynthetic phosphoglucosamine (Long, 1951).

Specificity. No NH_3 or other product giving a colour with Nessler's solution under the conditions described is evolved by acetylglucosamine, aspartic acid, glutamic acid, serine, threonine, citrulline, arginine, lysine, thymine, uracil, guanine, adenine, xanthine, allantoin, alloxan, creatinine, creatine, hydantoin, sarcosine, betaine, choline, ethanolamine, hyaluronic acid, or chitin. No NH_3 is evolved from a crude preparation of canavanine from jack bean meal. NH_3 is evolved in the presence of ammonium salts, glutamine, asparagine, nicotinamide, or urea. Interference by ammonium salts can be allowed for by a separate estimation using the Conway microdiffusion method (Conway, 1950). Glucosamine is decomposed at room and higher temperatures by half-saturated K_2CO_3 but not at all at 5°. A period of 4 hr. at 5° was found sufficient for complete diffusion of NH_3. Recoveries from glucosamine (100 μg. N) in this period at varying temperatures were 15% (35°), 4% (26°), 2% (20°), 1% (14°, using 500 μg. glucosamine N), and nil at 5°. Recoveries from galactosamine at 5° were also nil. The amide N of glutamine and asparagine appear as NH_3 after hydrolysis with N-H_2SO_4 at boiling point for 3 hr. (Pucher *et al.* 1935), and the same treatment was 80% effective with nicotinamide. Urea is only decomposed to the extent of 40% by this treatment. If the hydrolysed fluid is treated with urease before distillation and estimation of free NH_3, interference from this source will be eliminated. This is, however, only necessary in the presence of large amounts of urea as the yield on distillation is only about 0·5% of the urea N. Recoveries on distillation from solutions containing 1–2 mg. asparagine N or nicotinamide N are about 3% and from solutions containing 0·1–0·2 mg. glutamine N are about 10–20%. NH_3 is also formed by the distillation of some proteins under the conditions described. 10–20% of the amide N of glutenin and edestin were recovered in this way. After a preliminary hydrolysis with N-H_2SO_4 the N yields by Conway estimation at 5° and distillation were identical and agreed well with published figures for the amide N of these proteins. Thus the hydrolysis procedure seems equally effective in eliminating interference by both free and combined glutamine and asparagine.

Interference by mixtures of sugars and amino-acids occurs in this method as in the method of Elson & Morgan (1933) and its modifications. A direct comparison between the original method of Elson & Morgan and the method described in this paper was made. Using a 1·5 M solution of glucosamine hydrochloride the increase in apparent glucosamine caused by the presence of 2·5 M-lysine hydrochloride and 2·5 M-glucose was 18% for the first method and 5% for the second.

DISCUSSION

The method proposed has been used for more than a year in these laboratories for the analysis of chitin and products containing it. With relatively pure samples of chitin it has the advantage that decomposition of glucosamine on hydrolysis (Ogston & Stanier, 1950; Folkes, Grant & Jones, 1950) is simultaneously allowed for, if ammonia is not estimated at 5°, or measured if it is. It is not suggested that the method is completely satisfactory used alone, but in conjunction with one of the modifications of the Elson & Morgan method the two methods provide very useful safeguards against the acceptance of false results.

SUMMARY

1. A method for the estimation of amino sugars in the range 50–500 μg., by alkaline decomposition and estimation of the ammonia produced, is described.

2. Elimination of interference by other nitrogen-containing compounds is discussed.

It is a pleasure to thank Dr W. T. J. Morgan for the sample of galactosamine used in this work.

REFERENCES

Conway, E. J. (1950). *Micro-diffusion Analysis and Volumetric Error*. London: Crosby Lockwood.
Conway, E. J. & O'Malley, E. (1942). *Biochem. J.* **36**, 655.
Dische, Z. & Borenfreund, E. (1950). *J. biol. Chem.* **184**, 517.
Elson, L. A. & Morgan, W. T. J. (1933). *Biochem. J.* **27**, 1824.
Folkes, B. F., Grant, R. A. & Jones, J. K. N. (1950). *J. chem. Soc.* p. 2136.
Horowitz, H. N., Ikawa, M. & Fling, M. (1950). *Arch. Biochem.* **25**, 226.
Immers, J. & Vasseur, E. (1950). *Nature, Lond.*, **165**, 898.
Johnston, J. P., Ogston, A. G. & Stanier, J. E. (1951). *Analyst*, **76**, 88.
Long, C. (1951). Personal communication.
Lutwak-Mann, C. (1941). *Biochem. J.* **35**, 610.
McCrea, J. F. (1951). *Biochem. J.* **48**, xlix.
Markham, R. (1942). *Biochem. J.* **36**, 790.
Morgan, W. T. J. (1936). *Biochem. J.* **30**, 909.
Ogston, A. G. & Stanier, J. E. (1950). *Biochem. J.* **46**, 364.
Pucher, G. W., Vickery, H. B. & Leavenworth, C. S. (1935). *Industr. Engng Chem.* (Anal. ed.), **7**, 152.
Schloss, B. (1951). *Anal. Chem.* **23**, 1321.
Sideris, C. P., Young, H. Y. & Krauss, B. H. (1938). *J. biol. Chem.* **126**, 233.
Tracey, M. V. (1951). *Biochem. J.* **49**, xx.
Vanselow, A. P. (1940). *Industr. Engng Chem.* (Anal. ed.), **12**, 516.

The Metabolism of Methyl Carbamate

By E. BOYLAND and D. PAPADOPOULOS*
The Chester Beatty Research Institute, London, S.W. 3

(*Received* 3 *March* 1952)

Urethane or ethyl carbamate, which is carcinogenic (Nettleship & Henshaw, 1943), mutagenic (Vogt, 1948) and used in the treatment of leukaemia (Paterson, Ap Thomas, Haddow & Watkinson, 1946), is considered to be a radiomimetic agent. It is destroyed relatively slowly in the bodies of normal rats and mice and even more slowly by tumour-bearing animals (Boyland & Rhoden, 1949; Skipper *et al.* 1951).

Methyl carbamate is not a radiomimetic agent, being neither carcinogenic nor mutagenic (Larsen, 1947). The rate of destruction of methyl carbamate by rats has been followed by estimation of the concentration of the compound in the tissues at different times after injection.

EXPERIMENTAL

Estimation of methyl carbamate. Methyl carbamate was determined by the method used for urethane by Boyland & Rhoden (1949). The sample containing less than 0·40 mg. methyl carbamate in 2 ml. 2 N-KOH was placed in the outer ring of a Conway unit (Conway, 1947), the central compartment of which contained 1 ml. of 0·025 N-$K_2Cr_2O_7$ in 10 N-H_2SO_4. The unit, along with a blank unit in which M-K_2CO_3 replaced the 2 N-KOH solution, was heated to 37° for 16 hr. At the end of that time, KI was added to the $K_2Cr_2O_7$ and the liberated I_2 titrated with 0·1 N-$Na_2S_2O_3$ from a Conway burette. The difference between the titration figures of the KOH and K_2CO_3 units was proportional to the methyl carbamate present. As in the estimation of ethyl carbamate, the reaction was stoicheiometric and reproducible under constant conditions (as shown in Table 1). All determinations were carried out in duplicate, and duplicate sets of blank units with reagents alone were set up with each set of estimations. One ml. 0·1 N-$Na_2S_2O_3$ is equivalent to 1·87 mg. methyl carbamate.

Table 1. *Estimation of known quantities of methyl carbamate*

Methyl carbamate taken (mg.)	0·1 N-$Na_2S_2O_3$ used (ml.)	Titre as % of theory
0·05	0·0262	96
0·10	0·0547	103
0·20	0·1188	96
0·30	0·1551	98
0·40	0·2232	103

Table 2. *Excretion of methyl carbamate in urine of rats dosed with* 50 *mg./*100 *g. body weight*

Weight of rat (g.)	Methyl carbamate (mg.) excreted in urine: During first 4 hr. after injection	Methyl carbamate (mg.) excreted in urine: Between 4 and 24 hr. after injection	Percentage of dose excreted in 24 hr.
150	2·3	1·4	4·9
200	2·2	7·6	9·8
190	1·8	7·0	9·2
160	1·4	2·7	5·1

Urinary excretion of methyl carbamate by rats

Adult rats weighing between 100 and 200 g. were dosed by intraperitoneal injection with 50 mg./100 g. body weight of methyl carbamate. The animals were kept in metabolism cages and the urine collected for estimation of the excreted carbamate. The cages were washed 4 and 24 hr. after injection and the washings added to the collected urine.

* Present address: The Greek Anti-Cancer Institute, Athens.

The amounts of methyl carbamate excreted shown in Table 2 suggest that the drug is not concentrated in the kidney as the proportion of the dose of carbamate excreted in 24 hr. is of the same order as the proportion of urine excreted in 24 hr. to the total body water.

carbamate was then estimated in samples of the extracts.

The theoretical figures for the expected concentrations are based on the following assumptions: (1) the methyl carbamate is uniformly distributed in the body water (as indicated by the similar concentrations in blood and the tissues examined); (2) the body water forms two-thirds of the body weight of normal rats (Moulton, 1923) and 70% in tumour-bearing rats (an estimate based on scattered data in the literature); (3) the tissues examined contain 80% water; and (4) the rate of elimination of methyl carbamate at any time is proportional to the concentration in the body at that time.

Table 3. *The concentration of methyl carbamate in tissues of normal rats at different times after intraperitoneal injection of the drug*

Dose (mg./100 g. body wt.)	Time after injection (hr.)	Concentration of methyl carbamate found (mg./100 g.)			Theoretical expected concentration* (mg./100 g.)
		Blood	Lungs	Liver	
50	0	—	—	—	60
50	1	54	56	57	—
50	4	51	44	44	48
50	24	26	24	26	30
50	48	16	14	14	15
50	72	8	13	10	7
50	96	7	5	4	4
50	120	0	0	0	2
100	0	—	—	—	120
100	1	125	106	100	—
100	4	132	86	99	95
100	24	61	47	47	60
100	48	27	18	20	30
100	96	17	19	16	7
100	120	20	23	21	—
100	144	0	0	2	—

* Assuming that the concentration in the body falls by 50% in 24 hr.

Table 4. *The concentration of methyl carbamate in tissues of rats with Walker carcinomata at different times after intraperitoneal injection of the drug*

Dose (mg./100 g. body wt.)	Time after injection (hr.)	Concentration of methyl carbamate found (mg./100 g.)			Theoretical expected concentration* (mg./100 g.)
		Blood	Lungs	Liver	
50	0	—	—	—	57
50	4	67	61	70	—
50	24	50	43	40	38
50	48	20	12	23	28
50	72	—	—	—	19
50	96	—	—	—	14
50	120	16	10	4	—
50	144	—	—	—	7
100	0	—	—	—	114
100	4	138	77	108	—
100	24	56	40	53	85
100	48	67	48	55	57
100	96	31	27	28	28
100	120	27	10	24	21

* Assuming that the concentration in the body falls by 50% in 48 hr.

Distribution of methyl carbamate

Adult rats, with grafted Walker carcinomata, and normal rats were given doses of 50 and 100 mg./100 g. body weight of methyl carbamate by intraperitoneal injection and killed at different intervals after dosing. A sample of blood was collected and extracted with 2 volumes of 10% trichloroacetic acid. The lungs and a sample of liver were similarly extracted with the trichloroacetic acid. Methyl

The data on the distribution of methyl carbamate in rat tissues (Tables 3 and 4) show that the com-

pound is fairly quickly distributed in the body, but that the concentration in the tissues examined falls very slowly. It appears that the rate of elimination of methyl carbamate is such that the time required for the concentration to fall to half the maximum value is 24 hr. for normal rats and almost twice this time for tumour-bearing rats.

DISCUSSION

The data on elimination of ethyl carbamate (Boyland & Rhoden, 1949; Skipper *et al.* 1951) indicated that the time for 50% elimination was of the order of 12 hr. for normal rats or mice and 24 hr. for tumour-bearing animals. The present data indicate that methyl carbamate, like ethyl carbamate or ethanol, is not concentrated by the kidney, so that elimination must be mainly by metabolism. Methyl carbamate appears to be metabolized at about half the rate of ethyl carbamate.

The relationship of metabolism of the methyl and ethyl carbamates to their biological action is difficult to understand. If the radiomimetic action were due to hydrolysis of the carbamate, then the slower metabolism of methyl carbamate should be compensated for by increase of the dose. This, however, is not the case.

If the carcinogenic action of urethane were due to slow liberation of ethanol oxidized to acetaldehyde within the tissues then methanol would be expected to be as effective, as formaldehyde has been found to be mutagenic (Rapoport, 1946).

The discovery that ethyl carbamate is a much more effective inhibitor of transmethylation reactions than is methyl carbamate (McKinney, 1950) suggests that such inhibition may be concerned with the radiomimetic effects produced by urethane.

SUMMARY

1. Methyl carbamate, like urethane, does not appear to be concentrated by the kidney. Methyl carbamate is eliminated from the body by metabolism at about half the rate at which urethane is eliminated.

2. Tumour-bearing rats eliminate methyl carbamate only about half as fast as normal rats.

We should like to thank Mr R. W. White for his help with these experiments. This investigation has been supported by grants to The Royal Cancer Hospital and Chester Beatty Research Institute from The British Empire Cancer Campaign, The Jane Coffin Childs Memorial Fund for Medical Research, The Anna Fuller Fund, and The National Cancer Institute of the National Institutes of Health, U.S. Public Health Service.

REFERENCES

Boyland, E. & Rhoden, E. (1949). *Biochem. J.* **44**, 528.
Conway, E. J. (1947). *Microdiffusion Analysis and Volumetric Error*, revised ed. London: Crosby Lockwood.
Larsen, C. D. (1947). *J. nat. Cancer Inst.* **8**, 99.
McKinney, G. R. (1950). *J. Pharmacol.* **100**, 45.
Moulton, C. R. (1923). *J. biol. Chem.* **57**, 79.
Nettleship, A. & Henshaw, P. S. (1943). *J. nat. Cancer Inst.* **4**, 309.
Paterson, E., Ap Thomas, I., Haddow, A. & Watkinson, J. M. (1946). *Lancet*, **1**, 677.
Rapoport, I. A. (1946). *C.R. Acad. Sci., U.R.S.S.*, **54**, 65.
Skipper, H. E., Bennett, L. L., Bryan, C. E., White, L., Newton, M. A. & Simpson, L. (1951). *Cancer Res.* **11**, 46.
Vogt, M. (1948). *Experientia*, **4**, 68.

Sorbitol Antiketogenesis and the Dehydrogenation and Oxidation of Fatty Acids

By R. L. BLAKLEY*
Department of Biochemistry, Medical School, University of Otago, Dunedin, New Zealand

(*Received* 8 *January* 1952)

Sorbitol is remarkable for its ability to decrease the spontaneous ketogenesis of liver slices from fasted rats, being more efficient in this respect than related hexoses and most other compounds (Edson, 1936*b*; Blakley, 1951). During the investigation of this effect a dehydrogenase was found in rat liver which converts D-sorbitol to D-fructose in the presence of coenzyme I (CoI). The enzyme also converts L-iditol to sorbose, and iditol is as effective as sorbitol in diminishing spontaneous ketogenesis (Blakley, 1951).

A simple explanation of sorbitol antiketogenesis suggested by Edson (1936*b*) is that it results from competition between sorbitol and fatty acids for oxidative enzymes and coenzymes in the liver. When the course of sorbitol metabolism is taken

* Present address: National Institute for Medical Research, Mill Hill, London, N.W. 7.

into account it seems likely that Co I is the object of such competition between sorbitol dehydrogenation and fatty acid oxidation. Although Co I has not been proved essential for the oxidation of fatty acids, octanoate oxidation in washed suspensions of mitochondria is activated by Co I under some conditions (Lehninger, 1945; Lehninger & Kennedy, 1948; Kennedy & Lehninger, 1951). One possibility is that the $\alpha\beta$-dehydrogenation assumed to occur during β-oxidation of fatty acids, and demonstrated in the case of phenyl-substituted acids (Carter, Osman, Levine & Gamm, 1939; Mazza, 1935; Kuhn & Livada, 1933) requires Co I. This paper describes experiments performed with the object of testing this explanation of sorbitol antiketogenesis.

EXPERIMENTAL

Animals. Rats of the Wistar strain, fed as previously described (Blakley, 1951), were used.

Methods. Manometric measurements were made by standard methods. Homogenates were prepared with a stainless-steel instrument similar to the Potter-Elvehjem homogenizer, with a barrel 3 cm. in diameter and 15 cm. long.

Fatty acid oxidizing preparations. Two rat-liver preparations were used for the oxidation of fatty acids. The first was essentially the washed particulate material from a homogenate of rat liver in isotonic KCl (Lehninger, 1945). This is referred to throughout as the 'particulate preparation'. The second consisted mainly of washed mitochondria from rat liver, prepared by an abridged procedure of Lehninger (1949), in which the mitochondria are collected from a sucrose-containing homogenate at 2400*g* after agglutination with isotonic KCl. Considerable numbers of nuclei were present in this preparation, but it was substantially free from erythrocytes.

Sorbitol dehydrogenase. In order that fatty acid oxidation would not be unduly affected by impurities it was desirable to use a dehydrogenase preparation of greater purity than previously described. The enzyme was therefore prepared by an improved procedure as follows. After homogenizing the chilled livers of four rats for 1·5 min. in 6 vol. of ice-cold 0·01 M-potassium phosphate buffer, pH 7·8, in a chilled Waring Blendor, 2 N-HCl was added dropwise while the blending continued, until the pH had fallen to 4·7. The material was then immediately centrifuged at 2° in chilled centrifuge cups. After quickly adjusting the supernatant to pH 7·4, 0·25 vol. ethanol and 0·25 vol. $CHCl_3$ were added and the mixture shaken for 2 min. at room temperature and then immediately centrifuged at 2°. The supernatant, which should be light brown and was usually cloudy, was dialysed without delay against neutralized distilled water at 0° for 24 hr. The dialysed solution (about 200 ml.) was then adjusted to pH 5·0 when a red-brown precipitate usually formed, and was centrifuged off and discarded. The supernatant was treated successively with 30 and 50 ml. of alumina C_γ suspension (16·5 mg./ml.), the solid being centrifuged off after each addition. The sediment from the first centrifugation was used to obtain Co I-cytochrome *c* reductase (see below). To the almost colourless, clear supernatant from the second centrifugation were added 50 ml. of calcium phosphate suspension (24 mg./ml.), and the mixture centrifuged. The sorbitol dehydrogenase was eluted from the gel at room temperature with one 30 ml. and one 20 ml. portion of 0·01 M-Na_2HPO_4 containing $(NH_4)_2SO_4$ to 20% saturation. The dehydrogenase could be further concentrated by treating the dialysed eluate with 1 vol. of acetone at about −20°, rapidly centrifuging off the precipitate and dissolving it in cold 0·01 M-potassium phosphate buffer, pH 7·8. After dialysis against neutral distilled water at 2° the preparation lost little activity when kept at 2° for a week. The preparation was free from Co I-cytochrome *c* reductase and from colouring material and had an activity several times that of preparation *B* described previously (Blakley, 1951).

Coenzyme I-cytochrome c reductase. The O_2 uptake of earlier preparations of sorbitol dehydrogenase supplied with sorbitol, Co I and methylene blue, was not greatly increased by the addition of Straub's soluble diaphorase (Blakley, 1951). This was explained by the discovery that these dehydrogenase preparations contained an enzyme carrying out the oxidation of reduced Co I by cytochrome *c* and probably by methylene blue also. The enzyme responsible can be eluted from the first portion of alumina C_γ added in the procedure above, using 0·20 M-Na_2HPO_4. The purity of the reductase at this stage depends somewhat on the length of the previous treatment with ethanol-$CHCl_3$. A treatment for 2 min. necessary to eliminate cytochrome and haemoglobin, which are otherwise difficult to separate from sorbitol dehydrogenase, tends to produce larger amounts of brown contaminants in the reductase fraction. Accordingly, a treatment for 1 min. with ethanol-$CHCl_3$ was adopted in preparing reductase. The reductase can be further purified by adsorption on calcium phosphate gel at pH 7·0 and elution with 0·20 M-Na_2HPO_4. The two adsorption steps increased the activity about 12-fold, giving a faintly yellow, clear solution.

Analytical methods. Co I-cytochrome *c* reductase was determined by the method of Hogeboom (1949).

Octanoic acid was determined by the method of Lehninger & Smith (1948) with the following modification. The extractions were performed in rubber-stoppered test tubes and the lower aqueous layer removed after the extraction by means of a small wash-bottle head fitted into the test tube, as described by Weil-Malherbe & Bone (1949). Although the average recovery of octanoate was good, the accuracy obtained was not as high as reported by Lehninger & Smith.

Acetoacetate was determined manometrically (Edson, 1935).

Materials. Sorbitol and cytochrome *c* were the samples previously described (Blakley, 1951).

The octanoic acid used throughout this work was a product of Eastman Kodak Co. Other fatty acids were samples from British Drug Houses Ltd.

Adenosinetriphosphate (ATP) was obtained as the Ba salt from Boots Pure Drug Co. Ltd. The purity was 92–99% as judged by $P_{7\ min.}$, $P_{7\ min.}$/total P and P/N values.

The Co I used in these experiments was a product of Schwarz Laboratories Inc., New York, and assayed 50–60% by the dithionite method.

RESULTS

Effect of sorbitol on ketogenesis in rat-liver homogenates. As a preliminary to searching for a simplified cell-free system in which sorbitol antiketo-

genesis could be tested, it was desirable to examine the effect of sorbitol on ketone production in whole liver homogenates where none of the cell components had been discarded. Failure to produce antiketogensis in such a system could not be due to removal of components essential for the operation of the antiketogenic effect.

greater ketonuria than normal fasted rats. Although the precise mechanism of the effect is unknown, it seemed possible that the livers of rats so treated might provide more favourable material for the investigation of antiketogenesis. Accordingly, female rats, about 200 g. body weight, fasted 2–4 days and injected intraperitoneally with 35 mg.

Table 1. *Effect of co-factors on spontaneous ketogenesis in rat-liver homogenates*

(Female rat, fasted 4 days. Liver homogenized in 2 vol. 0·13 M-KCl containing 0·013 M-sodium phosphate buffer, pH 7·4. Flasks contained 0·13 ml. sodium phosphate buffer, pH 7·4 (0·013 M),* 0·03 ml. KCl (0·06 M), 0·60 ml. homogenate, water to give a final volume of 1 ml. and other components as below. Inseals contained 0·20 ml. 2 N-NaOH. Gas, O_2. Temp., 30°. Time, 75 min.)

Concentrations of components (M)				
Mg^{++}	Cytochrome *c* ($\times 10^{-5}$)	ATP	O_2 uptake (μmoles)	Acetoacetate formed (μmoles)
0·0000	0·8	0·0012	28·2	1·9
0·0023	0·8	0·0012	20·9	1·7
0·0046	0·8	0·0012	22·1	2·6
0·0046	0·0	0·0012	26·5	2·1
0·0046	0·4	0·0012	20·5	2·2
0·0046	1·3	0·0012	22·0	3·4
0·0046	0·8	0·0000	16·8	1·2
0·0046	0·8	0·0006	22·1	2·0
0·0046	0·8	0·0012	25·2	2·1
0·0046	0·8	0·0032	32·8	2·2

* The figures in parentheses indicate the concentration of the components in the complete reaction medium. This notation is used in Tables 1–11.

Although spontaneous ketogenesis in cell-free homogenates has not been reported previously, homogenates, in isotonic potassium chloride, of livers from fasted rats were found to produce large amounts of acetoacetate endogenously. A necessary precaution for this production of spontaneous ketogenesis in homogenates, however, is that dilution of the tissue suspension, during homogenizing or in mixing it with other solutions in the reaction vessel, must be minimized. The amount of acetoacetate produced varied considerably from one animal to another, but was practically independent of added magnesium ions, cytochrome *c* and ATP (Table 1), as expected in concentrated tissue suspensions of this type.

When sorbitol was added to these concentrated homogenates it produced no significant decrease in the spontaneous ketogenesis (Table 2). If Co I and nicotinamide were also added, sorbitol was vigorously oxidized as indicated by the increase in oxygen uptake, but the production of ketone bodies was still unaffected. The presence of added cytochrome *c*, ATP, magnesium ions, potassium chloride, acetyl phosphate, L-malate, cocarboxylase and yeast extract and combinations of these factors was unable to cause the appearance of any antiketogenic effect. Added fructose and glucose were similarly without effect on ketogenesis.

Janes & Brady (1949) reported that rats injected with nicotinamide during fasting exhibit a much

nicotinamide in saline each day of the fast, were used for the preparation of ketogenic liver slices and homogenates. Although it was confirmed that these animals exhibit high ketonuria, the spontaneous ketogenesis of liver slices and homogenates was not significantly greater than that for normal fasted animals. Moreover, in liver homogenates from such animals, sorbitol was again without effect on spontaneous ketogenesis.

Table 2. *Spontaneous ketogenesis in rat-liver homogenate in presence of sorbitol, glucose and fructose*

(Female rats, fasted 3 days and injected daily during fast with 35 mg. nicotinamide in 1·00 ml. normal saline. Livers homogenized in 2 vol. 0·13 M-KCl containing 0·013 M-sodium phosphate buffer, pH 7·4. Flasks contained 0·13 ml. sodium phosphate buffer, pH 7·4 (0·01 M), 0·06 ml. cytochrome *c* (6×10^{-6} M), 0·08 ml. $MgSO_4$ (0·012 M), 0·06 ml. ATP (0·0024 M), 0·03 ml. nicotinamide (0·01 M), 0·03 ml. Co I (2×10^{-4} M), 0·58 ml. homogenate and water to give a final volume of 1·00 ml. 30°. Gas, O_2. Time, 50 min.)

Additions	Acetoacetate formed (μmoles)
None	4·8
Sorbitol (0·05 M)	4·2
Fructose (0·05 M)	4·7
Glucose (0·05 M)	4·6

Rat-liver homogenates in 0·88 M-sucrose or isotonic potassium chloride rapidly oxidized added octanoate and pyruvate with the formation of

Table 3. *Acetoacetate production from added octanoate and pyruvate in rat-liver homogenate in presence of sorbitol*

(Female rats, fasted 24 hr. Livers homogenized in 2 vol. 0·88 M-sucrose. Flasks contained 0·30 ml. cytochrome *c* ($7·5 \times 10^{-6}$ M), 0·10 ml. ATP (0·001 M), 0·10 ml. $MgSO_4$ (0·0038 M), 0·30 ml. potassium phosphate buffer, pH 7·4 (0·015 M), 1·00 ml. homogenate and water to give a final volume of 4·00 ml. Inseals contained 0·20 ml. 2 N-NaOH. Gas, O_2. Temp., 30°. Time, 35 min.)

Substrate	Nicotinamide (0·01 M) and Co I (2×10^{-4} M)	Sorbitol (0·01 M)	O_2 uptake (μmoles)	Acetoacetate formed (μmoles)
None	Absent	Absent	12·9	4·3
Octanoate (0·001 M)	Absent	Absent	20·4	10·6
Octanoate (0·001 M)	Present	Absent	30·2	11·8
Octanoate (0·001 M)	Present	Present	30·8	9·7
Pyruvate (0·01 M)	Absent	Absent	19·6	8·4
Pyruvate (0·01 M)	Present	Present	24·9	8·7
Octanoate (0·001 M) + pyruvate (0·01 M)	Present	Absent	27·3	13·6
Octanoate (0·001 M) + pyruvate (0·01 M)	Present	Present	29·6	12·6
Octanoate (0·001 M) + α-ketoglutarate (0·001 M)	Present	Absent	28·3	10·2
Octanoate (0·001 M) + α-ketoglutarate (0·001 M)	Present	Present	31·5	8·4

acetoacetate. The production of acetoacetate under these conditions, too, was not significantly affected by the addition of sorbitol, even when Co I and nicotinamide were added to allow rapid sorbitol oxidation (Table 3). Similar results were obtained whether the livers of fasted rats or fed rats were used. Sorbitol is apparently devoid of antiketogenic action in liver homogenates even though it is effective in this respect in liver slices.

Coenzyme I *and the oxidation of octanoate by washed 'particulate preparations'*

In agreement with Lehninger & Kennedy (1948) it was observed that the washed particulate fraction of homogenates of liver in isotonic potassium chloride rapidly oxidizes octanoate (0·001 M) when supplied with magnesium ions, ATP and potassium chloride (0·05 M). Octanoate oxidation was also increased slightly by added cytochrome *c*. It was confirmed that with such preparations suspended either in water or in isotonic potassium chloride, Co I could replace ATP for octanoate oxidation, and in some cases Co I was found to increase octanoate oxidation even when ATP was already present (Table 4). As Lehninger was unable to find any further stimulation of octanoate oxidation by Co I when ATP was already present in the reaction medium, the question was further investigated. It was found that Co I consistently increased octanoate oxidation, both in the presence and absence of ATP, when liver preparations were obtained from fasted animals. When the liver of a recently fed animal was used, however, Co I could replace ATP as a factor for octanoate oxidation, but caused no further stimulation of oxidation if ATP were present.

It is difficult to establish whether the stimulatory effect of Co I on octanoate oxidation is due to the Co I as such or to other compounds present in the Co I preparation. In particular the effect might be due to adenosine-5-phosphate present in the preparation or formed enzymically from Co I during incubation. Such an explanation appears unlikely since the addition of extra ATP did not produce the same stimulation of octanoate oxidation (Table 4).

Table 4. *Effect of added Co* I *and other co-factors on octanoate oxidation with liver particulate preparations from fasted rats*

(Flasks contained 1·40 ml. of a water suspension of washed 'particulate preparation' from livers of fasted rats, 0·40 ml. octanoate (0·001 M), 0·10 ml. ATP (0·001 M), 0·15 ml. cytochrome *c* (10^{-5} M), 0·10 ml. $MgCl_2$ (0·004 M), 0·20 ml. potassium phosphate buffer, pH 7·4 (0·01 M), 0·10 ml. KCl (0·05 M), 0·50 ml. Co I ($7·8 \times 10^{-4}$ M), 0·13 ml. nicotinamide (0·01 M) and water to give a final volume of 4·00 ml. Inseals contained 0·20 ml. 2 N-NaOH. Gas, air. Temp., 38°. Time; 45 min.)

	O_2 uptake (μmoles)	Acetoacetate formed (μmoles)
Exp. 1		
Complete system	11·0	5·5
Octanoate omitted	2·9	0·4
ATP omitted	3·6	2·3
$MgCl_2$ omitted	2·1	1·2
Cytochrome *c* omitted	8·9	4·3
Nicotinamide omitted	10·9	4·8
Co I omitted	4·5	2·9
Co I and ATP omitted	0·2	1·2
Co I and nicotinamide omitted	4·9	2·7
Exp. 2		
Complete system	20·8	11·2
Co I omitted	12·7	7·4
Co I replaced by extra ATP (0·002 M)	12·0	6·7

Lehninger (1945) has stated that Co I could restore the activity of preparations aged 4–6 hr. In the present investigation difficulty was experienced in demonstrating such an effect of Co I because no decrease could be observed in the activity of washed

preparations suspended in water and kept at 0° for 6 hr. If the tissue was suspended in 8 vol. of water the activity immediately disappeared and could not be restored by CoI.

Despite the stimulatory action of CoI on octanoate oxidation in 'particulate preparations', CoI inhibited octanoate oxidation in preparations of mitochondria, both in presence and absence of ATP. The cause of this contrasting behaviour in the different preparations is not apparent. Recombination of the nuclear fraction of homogenates prepared in sucrose solution with the preparation of mitochondria produced a system which was similar to the 'particulate preparations' but in which CoI still inhibited fatty acid oxidation.

antiketogenic effect, both in the presence and absence of CoI and dehydrogenase (Table 5).

In order to test whether the interaction of fatty acid oxidation and sorbitol dehydrogenation occurred through some stage in the reoxidation of reduced CoI, CoI-cytochrome *c* reductase was added to the system with the hope of accelerating reoxidation of reduced CoI. The results obtained in such experiments, however, were essentially the same as those obtained in absence of added CoI-cytochrome *c* reductase.

In further attempts to produce modifications of the above experiments favourable to the demonstration of antiketogenesis, the following changes were made. First 'particulate preparations' were

Table 5. *Octanoate oxidation by washed liver 'particulate preparation' in presence of sorbitol, fructose, CoI and sorbitol dehydrogenase*

(Flasks were filled as in Table 4 except that CoI was omitted unless stated otherwise below. Octanoate (0·0025 M) present unless otherwise stated. Temp., 38°. Gas, air. Time, 45 min.)

Additions	O_2 uptake (μmoles)	Octanoate used (μmoles)	Acetoacetate formed (μmoles)
None	8·8	2·6	4·5
Sorbitol dehydrogenase (0·60 ml.)	10·0	3·8	6·4
Sorbitol dehydrogenase (0·60 ml.) + sorbitol (0·01 M)	12·6	3·5	7·5
CoI ($3·1 \times 10^{-4}$ M)	12·1	2·8	6·2
CoI ($3·1 \times 10^{-4}$ M) + sorbitol dehydrogenase	11·7	4·6	5·8
CoI ($3·1 \times 10^{-4}$ M) + sorbitol dehydrogenase (octanoate omitted)	3·0	—	0·1
CoI ($3·1 \times 10^{-4}$ M) + sorbitol dehydrogenase (0·60 ml.) + sorbitol (0·01 M)	18·7	3·2	6·7
CoI ($3·1 \times 10^{-4}$ M) + sorbitol dehydrogenase (0·60 ml.) + sorbitol (0·01 M) (octanoate omitted)	8·9	—	0·2
None	13·3	5·5	8·7
Fructose (0·01 M)	15·3	5·5	9·3
Fructose (0·01 M) + sorbitol dehydrogenase (0·60 ml.)	15·9	6·4	9·8
Fructose (0·01 M) + CoI ($3·1 \times 10^{-4}$ M)	18·1	6·6	8·2
Fructose (0·01 M) + CoI ($3·1 \times 10^{-4}$ M) + sorbitol dehydrogenase (0·60 ml.)	15·4	6·4	9·1

Sorbitol and octanoate oxidation in washed 'particulate preparations'

The washed 'particulate preparation' in which CoI was observed to produce some activation of octanoate oxidation promised to be a favourable system for the demonstration of antiketogenesis operating by way of competition for CoI. The effect of the sorbitol system on octanoate oxidation was accordingly tested in such systems, first in washed 'particulate preparations' from fed rats. The results, shown in Table 5, indicate that the sorbitol dehydrogenase preparation itself caused a slight stimulation of oxygen uptake, octanoate disappearance and even acetoacetate formation, but the further addition of CoI and sorbitol causing rapid oxidation of sorbitol, effected no inhibition of fatty acid oxidation. Sorbitol and dehydrogenase, added without CoI, were likewise unable to diminish acetoacetate formation or octanoate disappearance, even though the amount of CoI available would be limiting under these conditions. In a control experiment fructose likewise failed to show any

made from the livers of fasted rats to produce conditions in which CoI has its maximum effect on octanoate oxidation, and secondly stearic acid was oxidized instead of octanoic to approximate more closely to the probable precursor of acetoacetate in slices. In spite of these variations sorbitol dehydrogenation continued to show no sign of diminishing fatty acid oxidation or acetoacetate production (Table 6).

Sorbitol and the 'sparking' phenomenon

Lehninger & Kennedy (1948) have reported that when the particulate preparation from rat liver is washed with isotonic potassium chloride and finally suspended in a large volume of water, octanoate oxidation is not catalysed by the preparation unless an intermediate of the Krebs cycle is present in addition to the other normal co-factors. Later it was found that the oxidation of reduced CoI could replace the oxidation of Krebs-cycle intermediates for this 'sparking' of fatty acid oxidation (Kennedy & Lehninger, 1951). As it seemed possible that this 'sparking' of fatty acid oxidation by the oxidation

of reduced CoI might in some way relate to sorbitol antiketogenesis, attempts were made to produce 'sparking' of octanoate oxidation by sorbitol oxidation.

octanoate oxidation was effected by L-malate (Table 7). In such preparations sorbitol dehydrogenation was quite devoid of any activating effect on octanoate oxidation (Table 7).

Table 6. *Oxidation of stearate and of octanoate in presence of the sorbitol dehydrogenase system*

(Flasks were filled as in Table 4, except that octanoate or stearate were added as indicated below, and CoI was omitted unless otherwise stated. In Exp. 1 the 'particulate preparation' was obtained from a fasted rat, in Exp. 2 from a fed rat. Temp., 38°. Gas, air. Time, 45 min.)

Additions	O_2 uptake (μmoles)	Octanoate used (μmoles)	Acetoacetate formed (μmoles)
Exp. 1			
Octanoate (0·001 M)	4·2	2·1	2·1
Octanoate (0·001 M) + CoI ($3·1 \times 10^{-4}$ M)	7·9	3·5	3·6
Octanoate (0·001 M) + sorbitol dehydrogenase (0·90 ml.) + CoI ($3·1 \times 10^{-4}$ M)	9·5	3·7	5·2
Octanoate (0·001 M) + CoI ($3·1 \times 10^{-4}$ M) + sorbitol (0·01 M) + sorbitol dehydrogenase (0·90 ml.)	22·3	6·8	7·0
Exp. 2			
None	3·1	—	0·6
CoI ($3·1 \times 10^{-4}$ M) + sorbitol dehydrogenase (0·60 ml.)	4·1	—	0·4
CoI ($3·1 \times 10^{-4}$ M) + sorbitol dehydrogenase (0·60 ml.) + sorbitol (0·01 M)	9·0	—	0·6
Stearate (0·00025 M)	7·1	—	2·4
Stearate (0·00025 M) + CoI ($3·1 \times 10^{-4}$ M) + sorbitol dehydrogenase (0·60 ml.)	7·4	—	2·1
Stearate (0·00025 M) + CoI ($3·1 \times 10^{-4}$ M) + sorbitol dehydrogenase (0·60 ml.) + sorbitol (0·01 M)	15·5	—	2·1
Stearate (0·00025 M) + CoI ($3·1 \times 10^{-4}$ M) + sorbitol dehydrogenase (0·60 ml.) + fructose (0·01 M)	7·0	—	1·8

Table 7. *Oxidation of octanoate by suspensions of liver 'particulate preparations' of various dilutions in presence of the sorbitol dehydrogenase system and of malate*

(Flasks filled as in Table 4 except the octanoate, CoI and nicotinamide were absent unless stated otherwise below. Washed particulate preparation of varying dilutions was added as indicated. Temp., 38°. Gas, air. Time, part (1): 60 min.; part (2): 45 min.)

Washed particulate preparation	Additions	Octanoate (0·0025 M)	O_2 uptake (μmoles)	Octanoate used (μmoles)	Acetoacetate formed (μmoles)
Exp. 1					
1·10 ml. diluted 4-fold	None	Absent	1·0	—	0·4
	None	Present	10·1	—	5·8
1·65 ml. diluted 6-fold	None	Absent	0·4	—	0·6
	None	Present	2·0	—	1·3
	L-Malate (0·005 M)	Absent	6·8	—	0·5
	L-Malate (0·005 M)	Present	11·1	—	2·2
2·85 ml. diluted 10-fold	None	Absent	0·6	—	0·0
	None	Present	0·5	—	0·1
	L-Malate (0·005 M)	Absent	2·7	—	0·0
	L-Malate (0·005 M)	Present	3·4	—	0·6
Exp. 2					
2·12 ml. diluted 8-fold	None	Absent	1·4	—	0·9
	None	Present	0·6	0·0	0·4
	Sorbitol (0·01 M) + CoI ($3·1 \times 10^{-4}$ M) + sorbitol dehydrogenase + nicotinamide (0·01 M)	Absent	6·3	—	0·8
		Present	6·2	0·0	1·0

In these experiments difficulty was experienced in reproducing the 'sparking' phenomenon. It was found that while preparations in which the wet tissue was diluted with water about fourfold required no addition of a Krebs intermediate for rapid octanoate oxidation, at a tenfold dilution there was negligible octanoate oxidation even when Krebs-cycle intermediates were present. At intermediate dilutions only slight stimulation of

Anaerobic dehydrogenation of fatty acids

The experiments described above showed that in washed 'particulate preparations' sorbitol dehydrogenation was without influence on the overall oxidation of fatty acids. The possibility still remained that if sorbitol antiketogenesis were a result of competition for CoI, and there existed a particular step in fatty acid oxidation for which CoI

was specifically required, then it might be possible to demonstrate competitive inhibition of this isolated reaction by sorbitol in a simplified system. Such a Co I-dependent step occurring in the sequence of reactions involved in fatty acid oxidation is unknown, but the activating effects of Co I on octanoate oxidation are consistent with its existence. A Co I-dependent dehydrogenation of the fatty acid chain would be a reaction of the required type, and a search was therefore made for such a process.

Table 8. *Anaerobic dehydrogenation of octanoate in Thunberg tubes with washed 'particulate preparation' and various co-factors*

(The complete system consisted of 0·20 ml. octanoate (0·001 M), 0·05 ml. $MgCl_2$ (0·004 M), 0·05 ml. ATP (0·001 M), 0·05 ml. KCl (0·05 M), 0·07 ml. nicotinamide (0·01 M), 0·25 ml. Co I ($7·8 \times 10^{-4}$ M), 0·05 ml. methylene blue (0·006 %), 0·20 ml. neutral HCN (0·005 M), 0·10 ml. potassium phosphate buffer, pH 7·4 (0·01 M), 0·70 ml. washed 'particulate preparation' and water to give a final volume of 2·00 ml. 38°.)

	Reduction time (min.)
Exp. 1	
Complete system	2·8
Octanoate omitted	8·2
ATP omitted	14·7
Co I omitted	3·0
KCl omitted	3·0
$MgCl_2$ omitted	3·4
Nicotinamide omitted	2·7
Exp. 2. (KCl, $MgCl_2$ and nicotinamide omitted. Preparation obtained from liver of starved rat)	
Complete system	5·5
Octanoate omitted	15·0
Co I omitted	5·5
Exp. 3 (KCl, $MgCl_2$, nicotinamide and Co I omitted. Liver from fed rat. Reaction mixture also contained malonate, 0·01 M)	
Complete system	5·5
Octanoate omitted	∞
ATP replaced by yeast adenylic acid	∞

In Thunberg experiments fatty acids were incubated with washed 'particulate preparations' in the presence of Co I and with methylene blue as hydrogen acceptor. A rapid dehydrogenation of octanoate occurred under these conditions, but it was subsequently found that added Co I was without influence on the rate of the reaction (Table 8), even in liver preparations from fasted rats. Consequently, it was not possible to demonstrate an interaction between this dehydrogenation of fatty acids and sorbitol dehydrogenation. Nevertheless, the fatty acid dehydrogenation was briefly investigated, and the results are reported below.

Co-factors for fatty acid dehydrogenation. Table 8 shows that Co I, nicotinamide or magnesium sulphate were unnecessary for the reaction, and it was also independent of the electrolyte (potassium chloride) concentration. The only co-factor necessary was ATP and this requirement was specific; ATP could not be replaced by adenosine-3-phosphate. (Adenosine diphosphate and adenosine-5-phosphate were not available.)

Substrate and reaction rates. When a series of straight-chain fatty acids was tested with the washed 'particulate preparation' and ATP by the Thunberg technique, all the saturated acids from

Table 9. *Substrate specificity of the fatty acid dehydrogenase system of rat liver*

(Tubes contained fatty acid, 0·05 ml. ATP (0·001 M), 0·50 ml. potassium phosphate buffer, pH 7·4 (0·025 M), 0·05 ml. methylene blue (0·0125 %), 0·10 ml. neutral HCN (0·005 M), 0·10 ml. malonate (0·01 M), 0·60 ml. washed 'particulate preparation' and water to give a volume of 2·00 ml. In Exp. 2 the reaction volume was 4·00 ml., but concentrations of compounds other than fatty acid were kept the same as in (1). Temp. 38°.)

Fatty acid (0·001 M)	Reduction time (min.)
Exp. 1	
None	∞
Acetate	∞
Propionate	∞
Butyrate	Partial reduction in 60 min.
n-Pentanoate	19·0
n-Hexanoate	5·2
n-Heptanoate	7·0
n-Octanoate	5·5
Nonanoate	8·5
Decanoate	9·2
Laurate	Partial reduction in 60 min.
Myristate	Partial reduction in 60 min.
Palmitate	10·0
Stearate	11·0
Undecylenate	Partial reduction in 60 min.
Oleate	14·0
Exp. 2	
Fatty acid (0·00025 M)	
None	40·0
Octanoate	5·0
Laurate	6·2
Myristate	9·8
Palmitate	6·5
Stearate	5·8
Undecylenate	6·5
Oleate	8·7

C_4 to C_{18} were dehydrogenated (Table 9). The reaction proceeded most rapidly with hexanoate and the rate fell off quickly with shortening of the carbon chain. Acids with carbon chains shorter than that of butyrate were not attacked. Heptanoate, octanoate and nonanoate were attacked at about the same rate as hexanoate and decanoate at about half this rate. Acids of greater chain length than C_{10} reacted more slowly still, but stearate and palmitate reacted more rapidly than laurate and myristate. The slower reaction of the higher acids,

especially C_{12} and C_{14}, was apparently due to the surface effects of these compounds, for when the concentration of fatty acid was lowered from 0·001 to 0·00025 M their reaction rates were considerably increased. At this concentration laurate, palmitate and stearate reacted almost as rapidly as octanoate, while myristate reacted at approximately half this rate.

The only unsaturated acids tested were undecylenate and oleate, which reacted at rates similar to those of saturated acids of similar chain length. The participation of oleate in the reaction is significant and is discussed below.

methylene blue in absence of substrate without inhibiting dehydrogenation of fatty acids, the dehydrogenation was normally carried out in the presence of 0·01 M-malonate and 0·005 M-HCN.

Intracellular distribution of the enzyme system. When a homogenate of rat liver in 0·88 M-sucrose was fractionated by centrifuging according to the abridged method of Lehninger (1949), the fatty acid dehydrogenase activity was found only in the mitochondrial fraction (Table 11). The soluble fraction was difficult to test for activity because of the rapid reduction of the methylene blue by endogenous substrates.

Table 10. *Effect of malonate and cyanide on aerobic dehydrogenation of fatty acids*

(Tubes were filled as for Table 8, Exp. 1. Temp., 38°.)

	Reduction time (min.)
Complete system	2·8
Octanoate omitted	4·3
Complete system + 0·20 ml. neutral HCN (0·005 M)	4·0
Octanoate omitted; 0·20 ml. neutral HCN (0·005 M)	11·0
Complete system + HCN (0·005 M) + malonate (0·01 M)	3·8
Octanoate omitted; HCN (0·005 M) + malonate (0·01 M)	34·0

Stability of the dehydrogenase. The enzyme system responsible for the dehydrogenation was rather rapidly inactivated at the incubation temperature of 38°, so that after 60 min. no further reduction of the methylene blue occurred. This explains why slow dehydrogenations did not reach completion at all (Table 9).

Table 11. *Intracellular distribution of the enzyme system causing anaerobic dehydrogenation of fatty acids*

(Tubes were filled as for Table 9, Exp. 1 except that the homogenate fraction added was as indicated below. Temp., 38°.)

Homogenate fraction	Octanoate (0·001 M)	Reduction time (min.)
Mitochondria	Absent	∞
Mitochondria	Present	7·0
Nuclear fraction	Absent	∞
Nuclear fraction	Present	∞
'Soluble fraction'*	Absent	3·2
'Soluble fraction'*	Present	3·2
Mitochondria + nuclear fraction	Absent	∞
Mitochondria + nuclear fraction	Present	6·7

* Submicroscopic particles and soluble material.

Effect of inhibitors. The anaerobic dehydrogenation of fatty acids was not inhibited by 0·005 M-potassium cyanide, nor by 0·01 M-malonate (Table 10). Arsenite (0·003 M), dinitrophenol (0·00025 M), fluoride (0·01 M) and arsenate (0·005 M, tested in absence of phosphate) increased the reduction time two- to three-fold. Since malonate and cyanide increased the time for reduction of

DISCUSSION

The main object of the present investigation has been to determine whether the antiketogenic effects of sorbitol and iditol are explicable in terms of a competitive inhibition of fatty acid oxidation. Of the possible factors for which sorbitol dehydrogenation and fatty acid oxidation might compete, Co I and ATP seem the most likely. Co I is essential to the enzymic oxidation of sorbitol and L-iditol, and ATP is probably involved in the further metabolism of the fructose and sorbose produced respectively by the dehydrogenation of these compounds. It has been shown, on the other hand, that the presence of Co I or ATP is essential for fatty acid oxidation, and with liver preparations from fasted animals maximal oxidation was found to occur only when both Co I and ATP were supplied. A situation in which both Co I and ATP would limit fatty acid oxidation would arise if they were involved in a rate-limiting synthesis of Co II, and the latter were essential for some step in fatty acid oxidation.

The experimental results indicate, however, that under conditions where competition for Co I or ATP or both might occur, sorbitol dehydrogenation was without influence on fatty acid oxidation. Neither the complete system for sorbitol dehydrogenation, nor various combinations of its components, showed any consistent tendency to diminish the oxidation of octanoate or stearate in washed 'particulate preparations' from rat-liver homogenates.

An attempt has also been made to isolate some step of fatty acid oxidation which requires Co I, in

the expectation that such a reaction would be competitively inhibited by sorbitol dehydrogenation. This investigation revealed an anaerobic dehydrogenation of straight-chain fatty acids with 4 to 18 carbon atoms, but the essential co-factor proved to be ATP and not Co I, so that the reaction is not relevant to the antiketogenesis problem.

This dehydrogenation of fatty acids is of interest, however, since it seems to be different from the 'desaturation' reaction described by Lang (1939) and by Annau, Eperjessy & Felszeghy (1942, 1943). Hypoxanthine and adenosine-5-phosphate are the coenzymes for the desaturation reaction (Lang & Mayer, 1939*a*, *b*), in which an olefinic linkage is introduced into the centre of the carbon chain of higher fatty acids, and the purified enzyme is reported to require Co I (Burton, 1948). The dehydrogenation described here was activated by addition of ATP, but adenosine-5-phosphate would be formed under the experimental conditions. Properties of the dehydrogenation system which distinguish it from the desaturation system, however, are its apparent independence of Co I, its ability to attack fatty acids of short and intermediate chain length, its insensitivity to cyanide (cf. Lang & Mayer, 1939*a*) and its association with subcellular particles. Furthermore, oleic acid is readily dehydrogenated, whereas it inhibits the desaturation enzyme (Burton, 1948) and accumulates during stearate desaturation (Lang & Adickes, 1939).

It is possible that the anaerobic dehydrogenation of fatty acids described in these experiments represents the initial step or steps of the aerobic process of β-oxidation. Consistent with this suggestion are the requirement for ATP, the location of the enzyme system in subcellular particles (probably mitochondria), and the wide range of fatty acids attacked. If this is the case $\alpha\beta$-unsaturated acids may be produced in the reaction, although the unexpected participation of ATP may imply more profound changes in the structure of the fatty acid.

The role of Co I in fatty acid oxidation remains obscure, for although added Co I stimulated octanoate oxidation in particulate liver preparations, the dehydrogenation of fatty acids appeared to be independent of added Co I. It is clear that this does not eliminate the possibility that Co I is a cofactor in fatty acid dehydrogenation, for bound Co I is present in mitochondria (Cross, Taggart, Covo & Green, 1949); nevertheless, the dehydrogenation is apparently not the site of the action of added Co I. Added Co I may act by accelerating the synthesis of some other essential factor, or by supplying energy through its enzymic reduction and re-oxidation.

To return to the problem of antiketogenesis, another possibility investigated was that sorbitol dehydrogenation is able to initiate or accelerate fatty acid oxidation under conditions in which the 'sparking' effect of malate and other Krebs-cycle intermediates has been reported to occur. In these experiments the 'sparking' effect of malate could be observed only with 'particulate preparations' suspended in water to give tissue concentrations within rather narrow limits. Under conditions in which some 'sparking' by malate did occur, sorbitol dehydrogenation was ineffective as a 'sparker'. A possible explanation of the non-requirement of 'sparker' under most conditions by these preparations is that they contained traces of oxidizable substrates. The oxygen uptake of these preparations in absence of added substrate was very low, however, amounting to about 10% of that in the presence of fatty acid. Figures given by Lehninger & Kennedy (1948) for rat-liver preparations, and by Grafflin & Green (1948) for rabbit-kidney preparations which required 'sparking', show about the same oxygen uptake in absence of added substrate compared with that in presence of fatty acid. Furthermore, it was found that a fourth washing of the preparation with isotonic potassium chloride produced no change in the preparation as regards requirement of 'sparker'.

It has always been assumed that the ketone bodies produced in the diabetic or fasted animal are largely formed by hepatic oxidation of the fatty acids mobilized to the liver as carbohydrate reserves are depleted. Consequently, the antiketogenic effect has been sought by looking for a suppression of hepatic oxidation of fatty acids. But it now seems probable that antiketogenic agents inhibit the formation of acetoacetate arising, not from the oxidation of fatty acids, but from some other source, for the following reasons. Antiketogenic agents do not diminish the production of acetoacetate from fatty acids added to liver slices (Edson, 1936*b*; Weinhouse, Millington & Friedman, 1949). Furthermore, it has been found that in cell-free systems sorbitol dehydrogenation has no effect on fatty acid oxidation. Finally, although sorbitol and fatty acid oxidation proceed unaffected after the tissue has been homogenized, antiketogenesis no longer occurs. It is possible that absence of the antiketogenic effect in homogenates is due to destruction of essential factors, or that disorganization of the cell stops equilibration between bound mitochondrial Co I and Co I outside the mitochondria. Alternatively, disruption of the cell may be considered to dislocate a complex series of metabolic events with which ketogenesis is fundamentally concerned. The synthesis of fatty acids may be suggested as such a coordinated process which is disrupted by destruction of the cell, and which might be intimately connected with antiketogenesis.

In the liver of the fasted animal synthesis of long-chain fatty acids practically ceases (Masoro, Chaikoff, Chernick & Felts, 1950), a similar failure of

fatty acid synthesis being also observed in the livers of diabetic animals (Brady & Gurin, 1950; Chernick, Chaikoff, Masoro & Isaeff, 1950). The question arises, what is the fate, in the diabetic or fasted animal, of the metabolic intermediates which normally act as precursors of long-chain fatty acids? One possibility which must be considered is that they are stabilized as acetoacetate. In this event, that is, if ketone bodies are produced both by oxidation of fatty acids and from fatty acid precursors, antiketogenic agents may act by restoring fatty acid synthesis and consequently diminishing formation of acetoacetate from the precursors of fatty acids. Such a hypothesis is consistent with the facts discussed above, and offers an explanation of the fact that antiketogenic agents do not suppress more than 70% of acetoacetate formation and usually much less, and that their effect is rather variable (Blakley, 1951).

There remains the question of the manner in which an antiketogenic agent such as sorbitol could assist the restoration of fatty acid synthesis. Sorbitol antiketogenesis is attributable, at least in part, to the dehydrogenation of sorbitol, because sorbitol is more effective than fructose in diminishing acetoacetate production (Blakley, 1951). It is possible that the effect of sorbitol dehydrogenation is to furnish a direct and readily available source of energy through the reoxidation of reduced CoI, a process yielding two energy-rich phosphate bonds per molecule (Lehninger, 1949). Glycerol and L-iditol, which are strongly antiketogenic *in vitro*, and lactate, which is moderately so, may also act by providing a ready supply of energy in this way. Ethanol and malate, although dehydrogenated by liver enzymes requiring CoI, are not antiketogenic in rat-liver slices (Edson, 1936*a*, *b*), but this is explained by their failure to be oxidized appreciably under these conditions as shown by the figures for oxygen uptake. On such a hypothesis oxidation of reduced CoI is considerably more efficient than glucose or fructose oxidation in supplying energy for fatty acid synthesis in liver slices since sorbitol and iditol are more antiketogenic *in vitro* than glucose, fructose or sorbose. This may be explained in part by assuming that the oxidation of reduced CoI is in fact a good source of energy for synthetic reactions especially when it is rapidly formed by a vigorous dehydrogenation such as the oxygen uptake figures show sorbitol to undergo in liver slices. It may also be due in part to unfavourable conditions for glucose oxidation in the livers of diabetic or fasted animals (Stetten & Stetten, 1951). An alternative explanation of the effect of sorbitol dehydrogenation is that reduced CoI is used to reduce acetyl radicals ('active acetate') to fatty acid.

To test these hypotheses techniques are required which were outside the scope of the present investigation. If they are correct, the *in vitro* antiketogenic effect of sorbitol involves a fundamental change in the intermediary metabolism of fat which may operate in the intact animal in spite of the rapid conversion of sorbitol to glucose *in vivo* (Stetten & Stetten, 1951).

SUMMARY

1. Spontaneous ketogenesis has been demonstrated in liver homogenates of fasted rats, and has been found independent of added co-factors. Such spontaneous ketogenesis and the formation of acetoacetate from added substrates in homogenates is not affected by added sorbitol even when the latter is being rapidly oxidized.

2. For the activation of octanoate oxidation in washed mitochondria suspensions, coenzyme I (CoI) can replace adenosinetriphosphate (ATP); when livers from fasted rats are used for the preparation of mitochondria the activating effects of ATP and CoI are additive.

3. The oxidation of octanoate with acetoacetate formation in washed mitochondria suspensions is unaffected by the simultaneous dehydrogenation of sorbitol by purified dehydrogenase preparations. The results are unchanged by the presence or absence of CoI and of CoI-cytochrome *c* reductase.

4. In the presence of methylene blue and ATP, saturated fatty acids (C_4 to C_{18}), undecylenate and oleate are dehydrogenated anaerobically in suspensions of the washed particles from rat-liver homogenates. No other co-factors are required; adenosine-3-phosphate cannot replace ATP.

5. The anaerobic fatty acid dehydrogenase system is probably located in the mitochondria. It is not inhibited by 0·005M-hydrocyanic acid or 0·01M-malonate.

6. The significance of these results for theories of the mechanism of sorbitol antiketogenesis is discussed.

It is a pleasure to acknowledge my indebtedness to Prof. N. L. Edson whose advice and criticism were an invaluable aid. I am grateful also to the New Zealand Medical Research Council for a Fellowship during the period in which this work was performed.

REFERENCES

Annau, E., Eperjessy, A. & Felszeghy, O. (1942). *Hoppe-Seyl. Z.* **277**, 58.
Annau, E., Eperjessy, A. & Felszeghy, O. (1943). *Hoppe-Seyl. Z.* **279**, 66.
Blakley, R. L. (1951). *Biochem. J.* **49**, 257.
Brady, R. O. & Gurin, S. (1950). *J. biol. Chem.* **187**, 589.
Burton, K. (1948). *Nature, Lond.*, **161**, 606.
Carter, H. E., Osman, E., Levine, H. & Gamm, S. (1939). *J. biol. Chem.* **128**, xiii.
Chernick, S. S., Chaikoff, I. L., Masoro, E. J. & Isaeff, E. (1950). *J. biol. Chem.* **186**, 527.
Cross, R. J., Taggart, J. V., Covo, G. A. & Green, D. E. (1949). *J. biol. Chem.* **177**, 655.
Edson, N. L. (1935). *Biochem. J.* **29**, 2082.
Edson, N. L. (1936*a*). *Biochem. J.* **30**, 1855.
Edson, N. L. (1936*b*). *Biochem. J.* **30**, 1862.
Grafflin, A. L. & Green, D. E. (1948). *J. biol. Chem.* **176**, 95.
Hogeboom, G. H. (1949). *J. biol. Chem.* **177**, 847.
Janes, R. G. & Brady, J. (1949). *Amer. J. Physiol.* **159**, 547.
Kennedy, E. P. & Lehninger, A. L. (1951). *J. biol. Chem.* **190**, 361.
Kuhn, R. & Livada, K. (1933). *Hoppe-Seyl. Z.* **220**, 235.
Lang, O. St A. K. (1939). *Hoppe-Seyl. Z.* **261**, 240.
Lang, O. St A. K. & Adickes, F. (1939). *Hoppe-Seyl. Z.* **262**, 123.
Lang, O. St A. K. & Mayer, H. (1939*a*). *Hoppe-Seyl. Z.* **261**, 249.
Lang, O. St A. K. & Mayer, H. (1939*b*). *Hoppe-Seyl. Z.* **262**, 120.
Lehninger, A. L. (1945). *J. biol. Chem.* **161**, 413.
Lehninger, A. L. (1949). *J. biol. Chem.* **178**, 625.
Lehninger, A. L. & Kennedy, E. P. (1948). *J. biol. Chem.* **173**, 753.
Lehninger, A. L. & Smith, S. W. (1948). *J. biol. Chem.* **173**, 773.
Masoro, E. J., Chaikoff, I. L., Chernick, S. S. & Felts, J. M. (1950). *J. biol. Chem.* **185**, 845.
Mazza, F. P. (1935). *Arch. Sci. biol., Italy*, **21**, 320.
Stetten, M. R. & Stetten, D. (1951). *J. biol. Chem.* **193**, 157.
Weil-Malherbe, H. & Bone, A. D. (1949). *Biochem. J.* **45**, 377.
Weinhouse, S., Millington, R. H. & Friedman, B. (1949). *J. biol. Chem.* **181**, 489.

Sedimentation and Diffusion of Human Albumins

2. NEPHROTIC HUMAN ALBUMINS AT A LOW CONCENTRATION

By P. A. CHARLWOOD
Courtauld Institute of Biochemistry, Middlesex Hospital Medical School, London, W. 1

(*Received* 13 *February* 1952)

Marked deviations from normal in the quantitative distribution of the serum proteins of nephrotic cases have been demonstrated by several methods, notably electrophoresis (Longsworth, Shedlovsky & MacInnes, 1939; Longsworth & MacInnes, 1940; Luetscher, 1940; Thorn, Armstrong, Davenport, Woodruff & Tyler, 1945). The presence of abnormally large amounts of lipid (Longsworth & MacInnes, 1940; Thorn *et al.* 1945), indicated the possibility of qualitative changes also. A number of different approaches have been made to decide whether there are any such differences between normal and nephrotic serum proteins, or between these and nephrotic urinary proteins.

Hewitt (1927) was unable to differentiate between serum and urinary albumin by determinations of optical rotatory power and dispersion. Similarly, Widdowson (1933) found no differences in the optical rotation, specific refractive increments, or racemization curves. None of these properties would, however, be likely to depend very critically on molecular weight, unless the alteration in molecular weight were associated with the incorporation of material other than protein.

The finding of Alving & Mirsky (1936), that the 'albumin' fraction, separated by half-saturation of nephrotic sera with ammonium sulphate, had a low cystine content is almost certainly due to the presence, in the supernatant 'albumin' solution, of a large proportion of α-globulin rich in lipid. As Gutman (1948) pointed out, the incompleteness of such separations must be taken into account when assessing results obtained with the fractions. (See also Bradley & Tyson, 1948.)

As regards electrophoretic properties, Longsworth & MacInnes (1940) noted that the mobilities of the urinary albumins were lower than those of normal or the corresponding serum albumins. It was discovered by Luetscher (1939) that the two components which separated from both nephrotic serum and nephrotic urinary albumin during electrophoresis at pH 4 were in approximately the same relative proportions, but their ratio differed from that found with normal human albumin.

Somewhat similar phenomena have been observed by Hoch-Ligeti & Hoch (1948) during protracted electrophoresis at pH 8.

It is important to establish whether the molecular weights of nephrotic proteins are perceptibly abnormal. Although osmotic measurements by Widdowson (1933) failed to reveal any variation, Bourdillon (1939), also on the basis of osmotic measurements, claimed that the molecular weights of the serum proteins were higher, and those of the urinary proteins lower, than normal. Diffusion constants determined by Longsworth & MacInnes (1940) tended to support the contentions of Bourdillon (1939), as albumin from the urine possessed a higher diffusion constant than normal. Malmros & Blix (1946) measured the sedimentation constants of the urinary albumin in three cases, finding no detectable departure from their own normal value.

The initial concentration of protein in the urine samples varied within wide limits from one patient to another and often between samples from the same patient at different times. Consideration was given to the possibility of concentrating the urinary proteins by complete precipitation and subsequent solution in a reduced volume, by ultrafiltration (cf. Rigas & Heller, 1951), or by dialysis against gum arabic, polyvinyl pyrrolidone, or dextran solutions. As none of these methods was felt to be completely free from objection in the present instance, work was confined to samples of urine containing at least 0·5 % total protein, thus obviating the need for preliminary concentration. It did, however, mean that duplicate preparative runs were often essential, as with the sera.

Sedimentation measurements. The technique previously used (Charlwood, 1952) was adhered to, with one small exception, in spite of the appearance in the meantime of a potentially rather more accurate method of locating the peaks on the photographic plates (Kegeles & Gutter, 1951). The precision of this Fresnel interference method of Kegeles & Gutter (1951) decreases somewhat at the lower concentrations used here, the standard error reaching about 1 %. The minor alteration referred to above was the adoption of a null method in making rotor temperature measurements with the contact thermocouple and galvanometer.

Table 1. *Sedimentation constants of nephrotic human albumins*

Sample no.*	Buffer used in isolation	Solvent for sedimentation	Protein concn. (%)	$s_{20,w}$
S 1	Phosphate (I=0·1)	Phosphate (I=0·1)	0·25	4·21†
U 1	,, ,,	,, ,,	0·17	4·29†
S 2	Veronal (I=0·05)	0·2 N-sodium chloride	0·24	4·32‡†
U 2	,, ,,	,, ,,	0·19	4·30‡†
U 3	,, ,,	,, ,,	0·20	4·34
S 4	Veronal (I=0·1)	0·05 N-sodium chloride	0·21	4·24
U 4	,, ,,	,, ,,	0·17	4·32
S 5	,, ,,	,, ,,	0·17	4·30
U 5	,, ,,	,, ,,	0·19	4·30
S 6	,, ,,	,, ,,	0·20	4·26
U 6	,, ,,	,, ,,	0·21	4·27
S 7	,, ,,	,, ,,	0·17	4·23
U 7	,, ,,	,, ,,	0·22	4·31
S 8	,, ,,	,, ,,	0·26	4·24
U 8	,, ,,	,, ,,	0·19	4·31

* *S* and *U* refer respectively to serum and urinary albumins, whilst numbers indicate particular patients.
† Refrigeration used during these runs. ‡ Mean of two determinations.

Since neither a diffusion constant, nor a sedimentation constant, is alone sufficient for the unambiguous estimation of a molecular weight, it was considered desirable that both these quantities should be measured for the same preparations. Albumin was selected, as it constitutes the major portion of the urinary protein, it is relatively homogeneous, and it can be isolated electrophoretically without great difficulty.

EXPERIMENTAL

Electrophoretic separations were carried out exactly as described by Charlwood (1952). In several cases the albumin content of the serum was so low that duplicate preparative runs were necessary, the products being bulked prior to the subsequent analytical run. This procedure proved superior to the alternative of extending the length of a single run because, owing to the large amount of lipid-rich α-globulin, convections tended to develop in the ascending limb and were liable to prevent a complete separation.

Diffusion measurements. The apparatus of Creeth (1952) was used under the conditions described by Charlwood (1952). Unfortunately it was not possible to prepare and examine more concentrated solutions of nephrotic albumins, to investigate the distribution of bands in the interference pattern.

RESULTS

Sedimentation measurements. The values in Svedberg units, obtained for the sedimentation constants, corrected to water at 20°, are shown in Table 1. In carrying out the corrections the same value of $\bar{V}$, 0·733 has been used as for normal human albumin (see Charlwood, 1952). Even if the true value of $\bar{V}$ differs considerably from this, the magnitude of the correction is little affected. This is

in marked contrast to the dependence of molecular weight on the value of $\bar{V}$ used in calculations based on the combined sedimentation and diffusion results (see Discussion). All the values for the nephrotic albumins fall within the limits defined for normal albumins under similar conditions, i.e. $4{\cdot}27_5 \pm 0{\cdot}08$ (Charlwood, 1952). The mean value for the serum albumins is $4{\cdot}26_4$ and for the urinary $4{\cdot}30_4$. Statistical analysis shows that only in the latter case is there any likelihood of these mean values representing real departures from normal, the value of the probability lying between 0·03 and 0·1, depending on whether possible effects of concentration are ignored or approximately allowed for.

Table 2. *Diffusion constants of nephrotic human albumins*

Sample no.*	Buffer used in isolation	Solvent for diffusion	Protein concn. (%)	No. of experiments	$D_{20,w}$
U 3	Veronal ($I=0{\cdot}05$)	0·2 N-sodium chloride	0·20	4	6·62
S 4	Veronal ($I=0{\cdot}1$)	0·05 N-sodium chloride	0·21	4	6·29
U 4	" "	" "	0·17	4	7·04
S 5	" "	" "	0·17	3	4·85
U 5	" "	" "	0·19	4	6·63
S 6	" "	" "	0·20	4	6·09
U 6	" "	" "	0·21	4	6·23
S 7	" "	" "	0·17	4	5·78
U 7	" "	" "	0·22	4	6·50
S 8	" "	" "	0·26	4	6·02
U 8	" "	" "	0·19	4	6·51

* Sample numbers denote the same materials as those in Table 1.

Diffusion measurements. Table 2 gives the diffusion constants of the nephrotic albumins, reduced to water at 20° and expressed in terms of the unit of 10^{-7} cm.^{2} sec.$^{-1}$. In a previous paper it was shown that corresponding normal albumins have a mean diffusion constant of 6·32 (Charlwood, 1952). Taking into account the small, real differences which exist between normal preparations and the errors of measurements, on the basis of 95 % fiducial limits, values obtained by the present method and lying within the range 6·11–6·52 may be classed as normal. Of the five serum albumin values shown in Table 2 only one falls within this range, a second slightly below the lower limit, whilst the other three are definitely low. Of the six urinary albumins three gave high diffusion constants, two fall practically on the upper limit of the range and only one appears quite normal. In every case the diffusion constant of the serum albumin was lower than that of the corresponding urinary component.

DISCUSSION

Sedimentation results. Previous sedimentation experiments with nephrotic albumins are confined to oil-turbine ultracentrifuge measurements by McFarlane (1935*a*, *b*) and Malmros & Blix (1946). The question of the absolute accuracy of oil-turbine values has been discussed earlier (Charlwood, 1951, 1952). Since that work was completed evidence of further discrepancies between oil-turbine and other ultracentrifuge measurements has become available (Kegeles & Gutter, 1951). However, the chief concern here is the comparison of the behaviour of normal and pathological specimens under similar conditions.

Malmros & Blix (1946) quoted 4·58, 4·54 and 4·52, for three different urinary albumins, as agreeing with their normal human albumin value, but the protein concentrations were not specified. McFarlane (1935*a*, *b*) compared corresponding serum and urinary albumins, concentrating the latter by means of ammonium sulphate precipitation. There was some suggestion that sedimentation was respectively slower and faster than usual, but in practically every case the differences did not exceed the probable experimental error. Although this is interesting in view of the present results, the main conclusion must be that there is no really valid evidence for deviations from normal as regards sedimentation.

Diffusion results. The diffusion of albumin from one case of nephrosis was studied by Longsworth & MacInnes (1940). Otherwise the only work described on this subject is that of McFarlane (1935*a*, *b*) who observed the spreading of sedimenting boundaries. In two nephrotic sera the rate of spreading of the albumin boundary was abnormally high; but, since the '*X*' protein peak was not visible, this could be ascribed to the polydispersity of the material in the boundary under consideration. In one case the corresponding urinary protein showed about the same degree of spreading, whilst in the other it was lower, although still greater than normal. It does not seem possible to draw definite conclusions from McFarlane's observations, owing to the reservation expressed concerning the identity of the material responsible for the boundaries. Measurements of diffusion constants from ultracentrifuge patterns are prone to err on the high side should the material

be at all polydisperse and are liable to be influenced by vibrational disturbance. Consequently, reliance can be placed only on measurements made under conditions free from such objections.

The diffusion constants obtained by Longsworth & MacInnes (1940) for a normal human serum and a nephrotic urinary albumin, when corrected to 20°, become 6·39 and 6·72 respectively. Their normal value is consistent with determinations made by the present technique (Charlwood, 1952). In addition, their urinary albumin diffused more rapidly than normal, in accord with the conclusions based on Table 2.

Molecular weights. On the assumption that the present results may be used without appreciable error in place of $s^0_{20,w}$ and $D^0_{20,w}$ (Charlwood, 1952), molecular weights may be calculated, the partial specific volume, $\bar{V}$, being the only remaining factor needed. It would have been impossible to determine this quantity with the requisite accuracy from the small amounts of material isolated in this work. Consequently, for the purpose of comparison, it has been necessary to assume that $\bar{V}$ is the same as for normal albumin (cf. Longsworth & MacInnes, 1940). Unless a significant part of the pathological albumin molecule consists of material which is not protein, this assumption is probably justified. Then, taking $\bar{V} = 0{\cdot}733$, the molecular weights calculated for the nephrotic serum albumins range from the normal value of about 62 000 (Charlwood, 1952) to 80 000, whilst the urinary ones lie between normal and 55 000. The limitations implicit in these statements must be emphasized. The molecular weights apply only to the albumin fraction which is isolated by electrophoresis under the present conditions and they are conditional on the correctness of $\bar{V}$. It should be noted, however, that the type of error most likely to be incurred is that which would be occasioned if the molecules of pathological albumin, particularly the serum albumin, included lipid portions. Then $\bar{V}$ would actually be higher and the above molecular weight estimates would be too low.

The osmotic pressure measurements of Bourdillon (1939) gave a molecular weight of 72 000 for normal human albumin, 57 000–67 000 for different nephrotic urinary albumins and 99 000–122 000 for nephrotic serum albumins. Bourdillon was careful to point out that his 'albumin' was defined as that part of the protein which remained in solution after half-saturation with ammonium sulphate. As is now well known (Gutman, 1948), this fractionation is imperfect and the albumin from nephrotic serum is accompanied by much α-globulin. The inferences from Bourdillon's measurements are, therefore, somewhat inconclusive. The objection of possible contamination with α-globulin does not apply to the electrophoretically isolated materials, as the analytical experiments failed to reveal any globulin impurity. The degree of resolution was sufficiently high to have detected, in most cases, the proportion of globulin which would be required to account for high values of molecular weight.

The general conclusions are that the mean molecular weights of nephrotic serum albumins are often higher than normal, the urinary usually lower. Deductions which can be made from these observations depend on whether the serum (or urinary) albumin is polydisperse or of uniform molecular weight. The evidence of Luetscher (1939), Hoch (1948, 1950) and Hoch-Ligeti & Hoch (1948) from electrophoresis and the diffusion characteristics of normal human albumin (Charlwood, 1952) make the former alternative by far the more probable. The combination of techniques used here does not give any simple average of molecular weight of polydisperse material (Ogston, 1949).

It would be expected that any smaller molecules would pass more readily through the kidney unless specific effects predominated. This agrees with the observed lower mean molecular weight of the urinary albumins and also with the fact that the albumin to globulin ratio is higher in nephrotic urine than in serum (Malmros & Blix, 1946). However, Rigas & Heller (1951) found that it is apparently the globulin components which predominate in the small quantities of urinary protein encountered in normal subjects.

The further possibility, mentioned by McFarlane (1935*b*), that differences in the urinary protein might be due to chemical or biochemical action of constituents of the urine, whilst by no means excluded, has not been discussed, because the abnormally high molecular weights found for the serum albumins help to make such a hypothesis superfluous.

Thus, although the nephrotic albumins are definitely abnormal, the evidence is not sufficient to decide whether the cause lies in an unusual distribution of normal albumin components or in the presence of new components. Present techniques do not permit fractionation of the albumin. If this should become possible and the fractions be distinguishable immunologically, it might be feasible to demonstrate the existence, or otherwise, of abnormal molecules.

SUMMARY

1. The albumin fractions of a number of nephrotic sera and urine samples have been isolated electrophoretically.

2. Sedimentation constants for these preparations in the concentration range 0·2–0·3 % agreed with determinations previously made on normal human albumins.

3. In the Gouy diffusiometer the nephrotic serum albumins generally diffused more slowly, and the urinary albumins more rapidly, than normal.

4. It has been demonstrated that nephrotic albumins are abnormal. Probably the mean molecular weights of nephrotic serum and urinary albumins are respectively higher and lower than normal.

The author wishes to express his thanks to Prof. E. C. Dodds, F.R.S., for his help and interest. He acknowledges very gratefully the co-operation of Prof. J. R. Marrack, M.A., M.D., Prof. M. L. Rosenheim, M.D., F.R.C.P., members of their departments and Dr D. N. Baron in making available the clinical material. Helpful suggestions regarding the manuscript have been made by Dr J. M. Creeth. This work has been carried out during the tenure of an Imperial Chemical Industries Research Fellowship in the University of London.

REFERENCES

Alving, A. S. & Mirsky, A. E. (1936). *J. clin. Invest.* **15**, 215.
Bourdillon, J. (1939). *J. exp. Med.* **69**, 819.
Bradley, S. E. & Tyson, C. J. (1948). *New Engl. J. Med.* **238**, 223.
Charlwood, P. A. (1951). Thesis. University of London.
Charlwood, P. A. (1952). *Biochem. J.* **51**, 113.
Creeth, J. M. (1952). *Biochem. J.* **51**, 10.
Gutman, A. B. (1948). *Advanc. prot. Chem.* **4**, 155. New York: Academic Press Inc.
Hewitt, L. F. (1927). *Biochem. J.* **21**, 1109.
Hoch, H. (1948). *Biochem. J.* **42**, 181.
Hoch, H. (1950). *Biochem. J.* **46**, 199.
Hoch-Ligeti, C. & Hoch, H. (1948). *Biochem. J.* **43**, 556.
Kegeles, G. & Gutter, F. J. (1951). *J. Amer. chem. Soc.* **73**, 3770.
Longsworth, L. G. & MacInnes, D. A. (1940). *J. exp. Med.* **71**, 77.
Longsworth, L. G., Shedlovsky, T. & MacInnes, D. A. (1939). *J. exp. Med.* **70**, 399.
Luetscher, J. A., jun. (1939). *J. Amer. chem. Soc.* **61**, 2888.
Luetscher, J. A., jun. (1940). *J. clin. Invest.* **19**, 313.
McFarlane, A. S. (1935*a*). *Biochem. J.* **29**, 1175.
McFarlane, A. S. (1935*b*). *Biochem. J.* **29**, 1202.
Malmros, H. & Blix, G. (1946). *Acta med. scand.* Suppl. no. 170.
Ogston, A. G. (1949). *Biochem. J.* **45**, 189.
Rigas, D. A. & Heller, C. G. (1951). *J. clin. Invest.* **30**, 853.
Thorn, G. W., Armstrong, S. H., jun., Davenport, V. D., Woodruff, L. M. & Tyler, F. H. (1945). *J. clin. Invest.* **24**, 802.
Widdowson, E. M. (1933). *Biochem. J.* **27**, 1321.

Studies in the Biochemistry of Micro-organisms

89. METABOLIC PRODUCTS OF *PENICILLIUM MULTICOLOR* G.-M. AND P. WITH SPECIAL REFERENCE TO SCLEROTIORIN

By J. H. BIRKINSHAW
Department of Biochemistry, London School of Hygiene and Tropical Medicine, University of London

(*Received* 12 *March* 1952)

During the routine testing of a number of moulds for new metabolic products five strains of *Penicillium multicolor* Grigorieva-Manilova and Poradielova came under observation. Four of these five strains produced in the mycelium a yellow colouring matter which was extractable with light petroleum and which bore a strong resemblance to a chlorine-containing compound obtained from *P. sclerotiorum* van Beyma by Curtin & Reilly (1940) and named by them sclerotiorin(e). Sclerotiorin had already been obtained independently in these laboratories in 1940 by Dr R. F. Hunter from the mycelium of *P. sclerotiorum*. This finding was not recorded in the literature since publication was forestalled by Curtin & Reilly (1940), but there was no doubt from the source of the substance, its composition (particularly its chlorine content) and a comparison of properties that Dr Hunter's isolate was identical with sclerotiorin. Since Hunter's preparation was still available it was compared with the substance isolated from the various strains of *P. multicolor* and identity was established.

One of the strains of *P. multicolor* (no. N.R.R.L. 2324, Northern Regional Research Laboratory, Peoria, Illinois) afforded, in addition to sclerotiorin in the mycelium, a culture solution which on acidification to about 0·5N with hydrochloric acid gave a deposit of crystalline needles. This apparently new product, denoted as product I, was somewhat soluble in water but was reprecipitated on acidification. It had m.p. 110·5° and gave in aqueous solution a cherry-red colour with ferric chloride. From the analysis and determination of the equivalent the new product had the probable empirical formula

$C_{13}H_{16}O_6$. Extraction with ether of the filtrate obtained after precipitation of the above product afforded a second new product, product II, as pale yellow leaflets, softening at 128–132° and clearing at 133–134° after preliminary shrinking. In aqueous solution this product also gave a cherry-red colour with ferric chloride. Analysis indicated an empirical formula of $C_{13}H_{18}O_7$. These products were isolated only from the first batch of flasks of strain no. N.R.R.L. 2324. Since further supplies were unobtainable these substances could not be further investigated.

Advantage was taken of the fact that higher yields of sclerotiorin were obtainable from *P. multicolor* than had been recorded from *P. sclerotiorum*, to prepare a quantity of the material from the former species after selecting the highest yielding strain. It was thus possible to characterize the material more completely by the preparation of several crystalline derivatives and by further analyses of sclerotiorin itself and of the derivatives to advance a more firmly based empirical formula. Sclerotiorin was found to have a high optical rotation: $[\alpha]_D^{21°} + 500°$; other properties were in agreement with those described by Curtin & Reilly (1940). The empirical formula considered to be in best agreement with all the analyses is $C_{21}H_{23}O_5Cl$, although $C_{21}H_{25}O_5Cl$ is not completely excluded. Curtin & Reilly (1940, correction) ascribed to sclerotiorin the formula $C_{20}H_{21}O_5Cl$ on the basis of one analysis.

With 1 molecule of 2:4-dinitrophenylhydrazine, sclerotiorin forms a compound $C_{27}H_{27(29)}O_8N_4Cl$ which is probably a hydrazone. With hydroxylamine in excess, 2 mol. combine with 1 of sclerotiorine to give $C_{21}H_{23(25)}O_4N_2Cl$ which contains 1 mol. of water less than is required for a dioxime. Sclerotiorin reacts immediately with 1 mol. of ammonia in the cold with loss of 1 mol. of water yielding $C_{21}H_{24(26)}O_4NCl$. This product, and the alkaline-hydrolysis acid, $C_{10}H_{16}O_2$, had previously been obtained by Dr R. F. Hunter but were not investigated in detail. It appears probable that sclerotiorin contains one reactive aldehyde or keto group capable of forming a hydrazone or oxime; in the reaction with the second molecule of hydroxylamine and with ammonia some type of heterocyclic ring formation may be involved.

When sclerotiorin is warmed with dilute alkali, hydrolysis occurs and a colourless optically active monobasic acid, $C_{10}H_{16}O_2$, m.p. 92–92·5°, can be isolated in almost quantitative yield assuming 1 mol. from 1 mol. of sclerotiorin. This acid is doubly unsaturated and, from the fact that it adds on maleic anhydride, the double bonds appear to be conjugated. The other fission products were amorphous and have not yet been further examined. Ozonolysis of the unsaturated C_{10} acid affords in low yield an acid, $C_5H_6O_3$, which was identified as β-acetylacrylic acid, $CH_3CO.CH{=}CH.COOH$, by comparison with a synthetic specimen of the free acid and by comparing the respective 2:4-dinitrophenylhydrazones. An optically active aldehyde or ketone, $C_5H_{10}O$, also a product of ozonolysis, was isolated in good yield as the (+)-2:4-dinitrophenylhydrazone. Since this must have an asymmetric carbon it can have only one formulation namely $CH_3.CH_2.CH(CH_3).CHO$ which is 2-methyl-*n*-butanal, the aldehyde corresponding with the optically active (fermentation) amyl alcohol found in fusel oil. Badin & Pacsu (1945) have described the 2:4-dinitrophenylhydrazone of this compound; their values for melting point and optical rotation correspond closely with ours. On the basis of these facts the product of alkaline fission of sclerotiorin must have the constitution 4:6-dimethylocta-2:4-dienoic acid, $CH_3.CH_2.CH(CH_3).CH{=}C(CH_3).CH{=}CH.COOH$.

The reason for the low yield of β-acetylacrylic acid obtained on ozonolysis is no doubt due to the fact that it is susceptible to further oxidation at the double bond. In confirmation of this a small amount of a bisdinitrophenylhydrazone was isolated which, as a mixed melting point determination indicated, was in all probability derived from methylglyoxal. This would be one of the expected products of ozonolysis of β-acetylacrylic acid.

There is one discrepancy which remains to be explained. The C_{10} acid was found to yield 1·8 *C*-methyl groups per molecule whereas the postulated constitution has three such groups. The group $=CH.C(CH_3){=}CH$ is stated by Kuhn & L'Orsa (1931) to give 85% of the theoretical value for 1 *C*-CH_3 group. No evidence was available for the effect of the other two methyl groups present in the radical $CH_3.CH_2.CH(CH_3)$—. 2-Methyl-*n*-butanol (racemic) was therefore examined and found to yield only 37% of the theoretical value for 2 *C*-CH_3 groups. The total acetic acid formed from the C_{10} acid should therefore be 0·74 + 0·85 mol. = 1·6 mol., which is in fairly good agreement with the 1·8 mol. actually found.

The constitution of one fission product of sclerotiorin has thus been determined; further work is in progress to establish the structure of the rest of the molecule.

EXPERIMENTAL

Organism. Five strains of *P. multicolor* Grigorieva-Manilova and Poradielova were examined. These were obtained from Dr K. B. Raper of the Northern Regional Research Laboratory, Bureau of Agriculture, Peoria, Illinois, U.S.A., under the catalogue numbers N.R.R.L. 764, N.R.R.L. 2058, N.R.R.L. 2059, N.R.R.L. 2060 and N.R.R.L. 2324. Strain N.R.R.L. 2058 was received by us in July 1948, the remainder in December 1949. All the strains except N.R.R.L. 2324 are mentioned in *A Manual of the Penicillia* (Raper & Thom, 1949).

Medium. The usual Czapek-Dox medium of the following composition was employed: $NaNO_3$ 2·0 g., KH_2PO_4 1·0 g., KCl 0·5 g., $MgSO_4.7H_2O$ 0·5 g., $FeSO_4.7H_2O$, 0·01 g., glucose 50 g., water to 1 l. The medium was distributed in 1 l. conical flasks, 350 ml. per flask. The flasks were plugged with cotton wool and sterilized by steaming on three successive days. They were then inoculated from wort agar slope cultures and incubated at 24° for the prescribed period.

Characteristics of cultures. The five strains of *P. multicolor* (twenty flasks of each strain) were incubated for 17 days, when ten flasks of each strain were harvested. The remaining ten flasks of each strain were harvested after 24 days. The following appearances were noted.

N.R.R.L. 764. Considerable pigmentation and sporing (in some flasks only sparse). All heavily sporing flasks had a strongly curled mycelium forming an island in the centre of the culture fluid.

N.R.R.L. 2058. Pure white mycelium. Traces of colour round edges. Pale medium.

Isolation of sclerotiorin from mycelia. The mycelium from each group of flasks was ground in a mortar and extracted in a Soxhlet with light petroleum of b.p. 40–60°, the yield of solid crystalline material separating from the petrol being collected each day. The extraction was continued until the day's yield (6 hr.) was less than 1 g. The crude sclerotiorin extracted from the mycelium by petrol was orange-yellow in colour and had m.p. about 200°. The product from each strain was separately recrystallized once from methanol, and then consisted of bright yellow needles melting 3–4° higher than previously and in each case showing no depression when mixed with the sclerotiorin obtained by Hunter from *P. sclerotiorum.* The collected data are recorded in Table 1. The whole of the partially extracted mycelia was finally combined and re-extracted continuously with petrol to exhaustion. In this way a further 11·4 g. of sclerotiorin were obtained. The total yield of pure sclerotiorin obtained from the five strains was 30·0 g., although from one strain, N.R.R.L. 2058, the yield was nil. The best yield was obtained from strain N.R.R.L. 2060 where the crude yield was 15% of the weight of the dry mycelium, without taking into account the fact that the mycelium at this stage was not completely extracted. This is a great improvement on the yield of 2% obtained by Curtin & Reilly (1940) from *P. sclerotiorum.*

Table 1. *Data obtained on harvesting groups of ten flasks of* Penicillium multicolor *incubated at* 24°

Strain N.R.R.L.	Time of growth (days)	pH of culture fluid	Glucose in culture fluid (%)	Wt. of dry mycelium (g.)	Soxhlet yield of sclerotiorine (g.)	M.p. of recrystallized sclerotiorin (°)	Further yield of sclerotiorin from combined mycelia (g.)
764	17	4·0	0·20	32·4	0·8	204–205	
2058	17	3·7	0·81	42·2	Nil	—	
2059	17	4·0	0·60	37·7	3·6	202–203	
2060	17	4·4	0·71	41·6	6·3	204–205	
2324	17	4·9	0·63	32·8	2·7	205–206	11·4
764	24	4·6	Nil	32·3	0·9		
2058	24	4·3	0·21	47·6	Nil		
2059	24	4·5	0·10	38·7	2·8		
2060	24	5·1	0·21	43·6	6·6		
2324	24	5·2	0·18	32·0	2·4		

N.R.R.L. 2059. Strong sporing and colour.

N.R.R.L. 2060. Colour strong, less sporing. Strong brown coloured reverse.

N.R.R.L. 2324. White, wrinkled mycelium, a few showing colour. Colour round edges, strong brown reverse.

At the termination of the incubation period the culture fluid was filtered from the mycelium, which was dried in a vacuum oven. Glucose was determined polarimetrically in the filtrate, pH by indicator papers.

Reactions of the culture solution with aqueous $FeCl_3$ at 17 days were as follows: N.R.R.L. 764, solution intense emerald-green colour, very heavy precipitate not highly coloured; N.R.R.L. 2058, nil; N.R.R.L. 2059, almost immediate very heavy precipitate, pale buff; N.R.R.L. 2060, fair reddish-orange colour and slight precipitate; N.R.R.L. 2324, deep reddish-purple colour, overnight buff flocculent precipitate. Similar, perhaps somewhat more intense, reactions were obtained at 24 days.

On acidification with HCl the culture fluid of strain N.R.R.L. 2324 alone gave an immediate turbidity followed by a crystalline deposit consisting of bunches of colourless needles apparently slightly less in amount after 24 than after 17 days.

No antibacterial activity was shown by the culture fluid of any strain against *Micrococcus pyogenes* var. *aureus* (Oxford H strain) or *Escherichia coli* (National Collection of Type Cultures R 6).

Isolation of metabolic products from culture fluid of strain N.R.R.L. 2324

The total filtrate from the ten flasks of strain N.R.R.L. 2324 incubated for 24 days gave a reddish-purple colour with aqueous $FeCl_3$. It was acidified with 150 ml. of conc. HCl (diluted with 150 ml. of water) to render it about 0·5 N. Almost colourless needles of *product I* were slowly deposited. After being chilled overnight the crystals were collected, washed with 0·5 N-HCl and dried. The faintly yellow crystals, wt. 0·41 g. had m.p. 108°, raised on sublimation to 110·5°. They were somewhat soluble in cold, more readily in hot water. They were precipitated again from cold water on addition of HCl to 0·5 N. The aqueous solution gave a cherry-red colour with $FeCl_3$; the compound did not contain halogen (Beilstein test). Found (Weiler): C, 58·5; H, 6·4%. No nitrogen present. $C_{11}H_{14}O_5$ requires C, 58·4; H, 6·2; $C_{13}H_{16}O_6$ requires C, 58·2; H, 6·0%. Equivalent by titration: 0·0275 g. required 1·78 ml. 0·1 N-NaOH to neutralize to phenolphthalein; the end point was difficult to determine

owing to a yellow colour produced with alkali. Equivalent 146. $C_{11}H_{14}O_5$ (dibasic) requires 113. $C_{13}H_{16}O_6$ (dibasic) requires 134. When the titrated solution was acidified to 0·5 N with HCl the original acid crystallized.

The filtrate from the acidified culture solution was extracted by shaking with ether (1·5 l. + 1·0 l. + 1·0 l.). On evaporation of the solvent 2·35 g. of a semi-solid residue remained. The extracted liquor after neutralization no longer gave a red-purple colour with $FeCl_3$. The ether extract was treated with successive portions of hot water. Crystals were obtained from the aqueous solution on cooling (0·31 g.) and a further amount (0·17 g.) on acidification of the mother liquor. These fractions had m.p. 123–124° with preliminary softening. A third fraction (0·35 g.) had m.p. 121°. Crystallization from ethyl acetate-benzene afforded pale yellow plates or leaflets consisting of *product II*, m.p. 130–131°. After sublimation in high vacuum the substance began to melt at 128°, clearing at 135°. Recrystallized from ethyl acetate, it shrank at 126°, softened at 128–132°, clearing at 133–134°. This behaviour appears to be characteristic and not due to impurity. The cherry-red $FeCl_3$ colour reaction in aqueous solution was almost identical with that obtained from product I. The substance contained no chlorine (Beilstein test) or methoxyl group. Found (Weiler and Strauss): C, 54·6; H, 6·3; $C_{13}H_{18}O_7$ requires C, 54·5; H, 6·3.

Unfortunately these two products, obtained from the culture filtrate, were only present in minute amount or absent from subsequent batches, so that they could not be further studied.

Accumulation of stock of sclerotiorin. Strain N.R.R.L. 2060, which had been selected from the preliminary experiment as affording the best yield of sclerotiorin was used for the preparation of a supply of the material. The details as to medium, method of culture and harvesting were the same as described for the preliminary experiment. The dry mycelium was ground in a mortar and extracted to exhaustion with light petroleum (b.p. 40–60°) in a large percolator with reflux condenser attached. The daily yield was filtered off and fresh petrol was used to continue the extraction. By evaporation of the petrol to small volume further recoveries of impure sclerotiorin from solution were made. The data for two batches of flasks worked up in this manner are recorded in Table 2.

In an attempt to obtain supplies of the products I and II from the culture fluid of strain N.R.R.L. 2324, one batch of 100 flasks was inoculated with this strain. The culture solution gave practically no $FeCl_3$ reaction and the desired products could not be isolated, but the yield of sclerotiorin from the mycelium was over 40 g. The data for this batch are also recorded in Table 2. The overall yields of reasonably pure sclerotiorin from the three batches (neglecting the cruder material obtained by concentration of the petrol used for extraction) amounts to 12·3 % of the dry weight of the mycelium.

Table 2. *Data for two batches of strain N.R.R.L.* 2060 *and one of N.R.R.L.* 2324 *of* Penicillium multicolor

Batch	Strain N.R.R.L.	No. of flasks	Period (days)	Final pH of culture medium	Dry wt. of mycelium (g.)	Final glucose concn. (%)	Yield of crude sclerotiorine: Wt. (g.)	Yield of crude sclerotiorine: % of dry mycelium	M.p. of crude product (°)	Recovered from evaporation of petrol (g.)
1	2060	101	18	4·6	411	0·64*	47·2	11·5	200–2	2·6
2	2060	100	20	4·5	379	0·40*	51·2	13·5	200–2	3·2
3	2324	100	18	5·0	345	0·52†	41·5	12·0	199–201	2·8

* By polarimeter. † By Benedict method.

Sclerotiorin

A sample recrystallized from methanol was sublimed in high vacuum at 170–180°, bath temperature. The product had m.p. 205–206°, unchanged on a second sublimation.

Found (Weiler and Strauss): C, 64·6, 64·2, 64·2; H, 6·0, 6·1, 5·8; Cl, 10·2, 9·1, 9·2 %; mol.wt. (Rast) 400, 393. (Found (Imperial Chemical Industries, Welwyn): C, 64·1, 64·6; H, 6·2, 6·2; Cl, 8·9, 9·0. Calc. for $C_{21}H_{23}O_5Cl$: C, 64·5; H, 5·9; Cl, 9·1 %; mol.wt. 391. Calc. for $C_{21}H_{25}O_5Cl$: C, 64·2; H, 6·4; Cl, 9·0 %; mol.wt. 393. Optical activity: $[\alpha]^{21°}_{5790} + 549°$; $[\alpha]^{21°}_{D} + 500°$ (*c*, 1 in $CHCl_3$).)

Derivatives

2:4-*Dinitrophenylhydrazone.* 2:4-Dinitrophenylhydrazine (0·20 g.) was dissolved in ethanol (25 ml.) and sclerotiorin (0·40 g., 1 mol. propn.) was added, followed by two drops of conc. HCl. The solution became dark red. It was refluxed for 20 min. and allowed to cool. Crystals (rods) had already begun to separate from the boiling solution. The crystals, intense dark red in colour, almost purple in bulk, were collected, washed with ethanol and dried *in vacuo*. The 2:4-*dinitrophenylhydrazone*, wt. 0·47 g., did not melt up to 360°. (Found (I.C.I. Welwyn): C, 57·0, 56·9, 56·8, 56·9; H, 5·3, 5·2, 4·9, 4·9; N, 9·5, 9·3; Cl, 5·8, 6·2, 6·2 %. $C_{27}H_{27}O_8N_4Cl$ requires C, 56·8; H, 4·8; N, 9·8; Cl, 6·2. $C_{27}H_{29}O_8N_4Cl$ requires C, 56·6; H, 5·1; N, 9·8; Cl, 6·2 %.)

Compound with hydroxylamine. Sclerotiorin (0·25 g.) was dissolved in ethanol (10 ml.) by warming, and hydroxylamine hydrochloride (0·25 g.) was added. The mixture rapidly darkened to an almost black liquid. It was refluxed for 0·5 hr. and allowed to cool. As there was no separation of crystals it was poured into water, and the bulky dark-red flocculent precipitate was collected, washed with water and dried. The product (0·26 g.) had m.p. 135–148°. After being twice recrystallized from methanol-water (4:1 v/v) the product (0·12 g.) was in the form of clusters of microplates, deep orange in colour, and appeared homogeneous. The *product* shrank and darkened from 164°, softened about 170° to a black tar, but there was no definite melting point. (Found (Weiler and Strauss): C, 62·9, 62·0; H, 6·4, 6·2; N, 7·0; Cl, 8·8. $C_{21}H_{23}O_4N_2Cl$ requires C, 62·6; H, 5·75; N, 7·0; Cl, 8·8; $C_{21}H_{25}O_4N_2Cl$ requires C, 62·3; H, 6·2; N, 6·9; Cl, 8·8 %.)

Compound with ammonia. Sclerotiorine (1·0 g.) was dissolved by gentle agitation in conc. NH_3 (*d*, 0·880, 20 ml.). The deep red solution obtained (5 min.) was immediately diluted with water (100 ml.) and made faintly acid to Congo red with 2N-H_2SO_4. The red precipitate was collected, washed with water and drained. Without drying it was crystallized from methanol (40 ml.)-water (20 ml.). The bright red needles obtained (0·85 g., m.p. 228°, to a black tar), were recrystallized from the same solvent. The *product* then had m.p. 228–229° (decomp.). (Found (I.C.I. Welwyn): C, 64·8, 64·9; H, 6·1, 6·2; N, 3·5, 3·6; Cl, 9·1, 9·2%. $C_{21}H_{24}O_4NCl$ requires C, 64·7; H, 6·2; N, 3·6; Cl, 9·1; $C_{21}H_{26}O_4NCl$ requires C, 64·3; H, 6·7; N, 3·6; Cl, 9·05%.)

Degradation

Alkaline hydrolysis. Isolation of C_{10} acid. Sclerotiorin (5 g., the crude petrol extract is quite satisfactory) was suspended in N-NaOH (500 ml.) and the mixture was warmed on the water bath to 50–55° with frequent shaking and maintained for about 0·5 hr. All the solid dissolved, forming a dark yellow-brown solution. The solution was cooled to 10° and 2N-H_2SO_4 (250 ml.) was added slowly with shaking and cooling so that the temperature did not rise above 15°. Then a slight excess of acid was added until the mixture was acid to Congo red. When the precipitate had settled the mixture was filtered. The solid was a light chocolate-brown powder when dry, wt. 2·63 g.

The solid was extracted with cold light petroleum (b.p. 40–60°, 4 × 50 ml.), filtering from the brown amorphous material. The crystalline orange-coloured residue (wt. 1·70 g.) left on removal of the petrol was recrystallized from methanol (20 ml.) + water (20 ml.) to give a slightly coloured product (1·55 g.) of m.p. 91–92°. This was dissolved in NaOH (0·4N, 25 ml.) and the solution was treated with decolorizing carbon (1 g.). On acidification with HCl the C_{10} *acid* formed a crystalline precipitate of colourless rods, m.p. 92–92·5°, from which chlorine was absent. (Found (Weiler and Strauss): C, 71·7; H, 9·1; *C*-CH_3, 16·1%. $C_{10}H_{16}O_2$ requires C, 71·4; H, 9·6, mol.wt. 168, for 1 *C*-CH_3, 8·9%, for 2, 17·9%, for 3, 26·8%.)

Equivalent. 0·5313 g. of the acid required 31·6 ml. of 0·1N-NaOH for neutralization to phenolphthalein, giving an equivalent of 168.

Optical rotation. $[\alpha]_{5461}^{20°} + 82·7°$, $[\alpha]_{5790}^{20°} + 71·4°$ (*c*, 4 in ethanol).

Derivatives of C_{10} acid

Hydrogenation. Adams's catalyst (PtO_2, 0·1000 g.) was hydrogenated at room temperature in ethanol (50 ml.) and required 11·12 ml. of H_2. The C_{10} acid (0·4965 g.) was then added and the hydrogenation continued. The uptake of H_2 was slow and practically ceased after 40 min. when 52·4 ml. (uncorr.) of H_2 had been absorbed. A further amount (0·1000 g.) of Adams's catalyst was added without prior reduction and the hydrogenation continued. The uptake was now rapid and reached constancy in 20 min. when a further 93·4 ml. (uncorr.) of H_2 had been absorbed (after allowing for catalyst uptake). The total uptake of H_2 at N.T.P. by the acid was 132 ml. For an uptake of four atoms of H 0·4965 g. of a compound $C_{10}H_{16}O_2$ requires 132 ml.

The solution was filtered from the catalyst and made up to 100 ml. with ethanol for determination of optical rotation, using a 4 dm. tube. $[\alpha]_{5461} + 22·0°$ in ethanol (*c*, 0·5).

On evaporation of the ethanol *in vacuo* a pale amber-coloured oil, consisting of the C_{10} *tetrahydro acid*, remained (0·494 g.). It was dried to constant weight over P_2O_5 for analysis. (Found (Weiler and Strauss): C, 69·9; H, 11·6; $C_{10}H_{20}O_2$ requires C, 69·7; H, 11·7% mol.wt. 172.)

Equivalent. The hydrogenated acid (0·4137 g.) in 5 ml. of ethanol was titrated with 0·1N-NaOH. It required 22·70 ml. (corr. for ethanol blank). Equivalent, 182, indicating a monobasic acid if $C_{10}H_{20}O_2$.

p-*Bromophenacyl ester.* The above neutralized solution was evaporated *in vacuo* to about 5 ml. and refluxed with *p*-bromophenacylbromide (0·634 g.) in ethanol (30 ml.) for 1 hr. A trace of sediment was filtered off. On chilling the solution, crystals (shining leaflets) appeared which redissolved at room temperature. The chilled solution was filtered quickly giving 0·20 g. of plates, m.p. 40–55°. The product was dissolved in light petroleum, filtered from a little insoluble matter and recrystallized from 90% aqueous methanol, giving colourless plates, m.p. 41°, of the p-*bromophenacyl ester.* (Found (Weiler and Strauss): C, 58·8; H, 6·8; Br, 21·3; $C_{18}H_{25}O_3$ Br requires C, 58·55; H, 6·8; Br, 21·6%.)

Diels-Alder product from C_{10} acid. The C_{10} acid (0·3 g.) and maleic anhydride (0·2 g.) were fused together (without solvent) and maintained at 100–110° for 2 hr. The viscous oily product was taken up in a little dry ether. On evaporation of the ether a crystalline mass remained. This was redissolved in dry ether, filtered, and an equal volume of light petroleum (b.p. 40–60°) was added. Crystals of the addition *product* slowly deposited, of m.p. 144–152°, after three recrystallizations 147–148°, and on final sublimation 149–150°. (Found (Weiler and Strauss): C, 62·4; H, 6·5; $C_{14}H_{18}O_5$ requires C, 63·1; H, 6·8.) This indicates that the acid contains a conjugated double-bond system.

Degradation of C_{10} acid by ozonolysis

The C_{10} acid (0·50 g.) was dissolved by shaking in water (50 ml.) containing $NaHCO_3$ (0·50 g.). Ozonized oxygen was passed through the solution at room temperature, the outflow being bubbled through Brady's reagent (2:4-dinitrophenylhydrazine (0·3%) in 2N-HCl). A yellow precipitate began to form in the bubbler and the reaction mixture became slightly turbid. The ozone was stopped after 2·5 hr. but the O_2 stream was continued for a further 1 hr. After standing overnight, the pH was 8·0–8·4. The solution was extracted with ether (2 × 25 ml.). The ether extract gave only a small amount of material precipitable with Brady's reagent; this was not further examined. Air was bubbled through the aqueous layer to remove traces of ether and then N-HCl (5·95 ml.) was added to take up all the alkali. The white crystalline precipitate produced (0·07 g.) had m.p. 92–93° and was unchanged C_{10} acid.

The filtrate from the acid was evaporated *in vacuo* and the distillate was titrated. The operation was repeated after addition of water (50 ml.) up to four times in all. The titrated volatile acids amounted to 24·83 + 5·20 + 0·98 + 0·32 = 31·3 ml. of 0·1N. The non-volatile residue in the flask was titrated and found to require 5·31 ml. of 0·1N-NaOH. The equivalent amount of HCl was added and the solution was evaporated to dryness *in vacuo*. The residue was refluxed for a few minutes with 100 ml. then 50 ml. of ether. On evaporation of the ether a crystalline solid mixed with oil was obtained, of wt. 0·08 g. The mixture was fractionated from ether-light petroleum. Some of the fractions were oily, but from the best fraction about 20 mg. were picked out and

sublimed at water-pump vacuum. This product shrank slightly at 110°, softened at 120–122° and melted at 123–124°, showing a crystalline sublimate in the melting point tube. (Found (Weiler and Strauss): C, 52·2; H, 5·1; calc. for $C_5H_6O_3$: C, 52·6; H, 5·3 %.) A sample of β-acetylacrylic acid was prepared synthetically from laevulic acid via β-bromolaevulic acid according to the method of Wolff (1891). After crystallization from benzene and sublimation at water pump vacuum the synthetic product showed the following behaviour in the melting point tube: shrinks 115–120°, softens 122°, melts 124–125°. A mixture of the ozonolysis and synthetic acids behaved as follows: shrinks 110°, softens 122°, melts 124–125°. From the fact that there was no depression the conclusion was drawn that the acid produced by ozonolysis was acetylacrylic acid. This conclusion was confirmed by preparation of the dinitrophenylhydrazone.

2:4-*Dinitrophenylhydrazone of ozonolysis acid.* The residues from the crystallization of the above acid were dissolved in water and treated with Brady's solution (50 ml.). After 2 days, the bright yellow precipitate was collected and dried. Wt. 0·06 g., m.p. 263–264° (decomp.). On recrystallization from dioxan crystalline aggregates consisting of microrods of the 2:4-*dinitrophenylhydrazone* separated (0·03 g.), m.p. 263–264° (decomp.), darkening and shrinking from 260°. (Found (Weiler and Strauss): C, 45·0; H, 3·5; N, 19·2; calc. for $C_{11}H_{10}O_6N_4$: C, 44·9; H, 3·4; N, 19·1 %.) A sample of the 2:4-dinitrophenylhydrazone was prepared in like manner to the above from synthetic β-acetylacrylic acid. After recrystallization from dioxan it had m.p. 263–264° (decomp.) with darkening and shrinking from 260°. A mixture of this substance with the dinitrophenylhydrazone derived from the ozonolysis product showed no depression in melting point.

2:4-*Dinitrophenylhydrazone in bubbler.* The yellow solid which had been precipitated in the bubbler containing Brady's reagent was collected (wt. 0·44 g.) and recrystallized first from ethanol and then twice from light petroleum (b.p. 80–100°), filtering from a little insoluble matter. The melting point of the main product was then constant at 133·5°. (Found (Weiler and Strauss): C, 49·8; H, 5·3; N, 21·0; calc. for $C_{11}H_{14}O_4N_4$ (from $C_5H_{10}O$): C, 49·6; H, 5·3; N, 21·0 %.)

Optical activity: $[\alpha]^{19°}_{5461}$ +44·2°, $[\alpha]^{19°}_{5790}$ +38·0° (*c*, 1 in acetone). Badin & Pacsu (1945) give for the 2:4-dinitrophenylhydrazone of (+)-2-methylbutanal-1 the values: m.p. 132·5–133°, $[\alpha]_D$ +32·1° in acetone (*c*, 4·99). The petrol-insoluble material from the bubbler was an orange powder. The amount was too small for recrystallization. It gave an intense blue-violet colour when dissolved in ethanol and treated with a drop of dilute NaOH indicating that it was a bisdinitrophenylhydrazone. It had m.p. 300° (decomp.) and showed no depression when mixed with authentic methylglyoxal bis 2:4-dinitrophenylhydrazone.

Carbon-methyl determination on 2-methylbutanol. The determination was made on a sample of racemic 'active amyl alcohol' (β-methylbutanol) obtained from British Drug Houses. (Found (Weiler and Strauss): *C*-CH_3 12·5 %. Theor. for $C_5H_{12}O$ (with 2 *C*-CH_3): *C*-CH_3 34·0 %.) The value found is thus 37 % of theoretical.

All melting points are uncorrected.

SUMMARY

1. Five strains of *Penicillium multicolor* Grigorieva-Manilova and Poradielova were investigated. From the mycelium of four of these strains sclerotiorin was isolated in considerably higher yield than had been obtained by Curtin & Reilly (1940) from the mycelium of *P. sclerotiorum* van Beyma.

2. From the analysis of sclerotiorin itself and its derivatives the most probable empirical formula is $C_{21}H_{23}O_5Cl$. It has $[\alpha]_D^{21°}$ + 500° in $CHCl_3$ (*c*, 1).

3. When sclerotiorin is warmed with dilute aqueous sodium hydroxide it is hydrolysed giving a crystalline doubly unsaturated monobasic acid, $C_{10}H_{16}O_2$. The acid gives rise to (+)-2-methyl-*n*-butanal and β-acetylacrylic acid on ozonolysis, indicating that the acid has the constitution (+)-4:6-dimethylocta-2:6-dienoic acid.

4. In addition, two new products were isolated from the culture fluid of one strain of the fungus. These two substances, product I, m.p. 110·5°, and product II, m.p. 133–134°, after softening at 128–132°, both gave in aqueous solution a cherry-red colour with ferric chloride. From the analyses probable empirical formulae were deduced as follows: product I, $C_{13}H_{16}O_6$; product II, $C_{13}H_{18}O_7$.

I wish to thank Messrs Imperial Chemical Industries Ltd., Butterwick Research Laboratories, The Frythe, Welwyn, for carrying out many of the elementary analyses.

REFERENCES

Badin, E. J. & Pacsu, E. (1945). *J. Amer. chem. Soc.* **67**, 1352.

Curtin, T. P. & Reilly, J. (1940). *Biochem. J.* **34**, 1419.

Kuhn, R. & L'Orsa, F. (1931). *Z. angew. Chem.* **44**, 847.

Raper, K. B. & Thom, C. (1949). *A Manual of the Penicillia.* London: Baillière, Tindall and Cox.

Wolff, L. (1891). *Liebigs Ann.* **264**, 229.

Phosphates of Brain during *in vitro* Metabolism: Effects of Oxygen, Glucose, Glutamate, Glutamine, and Calcium and Potassium Salts

By H. McILWAIN
Biochemical Laboratories, Institute of Psychiatry (British Postgraduate Medical Federation, University of London), Maudsley Hospital, S.E. 5

(*Received* 21 *January* 1952)

It has been reported previously from this laboratory that labile phosphates can be resynthesized by portions of cerebral tissue suspended in suitable fluid media *in vitro* (McIlwain, Buchel & Cheshire, 1951). In the present paper the phosphates are further characterized and shown to include substances with the properties of adenine polyphosphates. Evidence was presented previously which suggested that concentrations, in the tissue, of the labile phosphates or of their breakdown products, conditioned the rates of overall processes such as respiration or glycolysis. To pursue this theme the effects on the phosphates of altering some constituents of the suspending media have been examined, choosing constituents which are known markedly to affect respiration and glycolysis. Oxygen, glucose, glutamate and glutamine (see Weil-Malherbe, 1938; Stern, Eggleston, Hems & Krebs, 1949), and calcium and potassium salts (see Dickens & Greville, 1935; Canzanelli, Rogers & Rapport, 1942) have now been investigated in this way. The phosphates were found to be affected by all these substances, in a fashion which shows an interesting pattern of relationships to the concomitant changes in respiration and glycolysis.

EXPERIMENTAL

The salines were based on those described by McIlwain (1951*a*), and McIlwain *et al.* (1951). Any departures from these are described in the experiments concerned. Slices of cerebral cortex from guinea pigs were the main experimental material and were handled, and inorganic phosphates determined, according to McIlwain *et al.* (1951). Glutamate solutions were made by taking the L(+)-acid to pH 7·2 with NaOH.

Possible interference by phosphopyruvic acid in determination of phosphocreatine. Characterization of one of the labile phosphates resynthesized *in vitro* as creatine phosphate, depended previously on (*a*) its precipitation as Ca salt at pH 9 by 80% but not by 10% ethanol, (*b*) its determination as inorganic phosphate by Fiske & Subbarow's conditions (see Hawk, Oser & Summerson, 1947) but not by Lowry & Lopez's (1946) conditions, (*c*) the approximate equivalence between the quantity of creatine and of labile phosphate precipitated as described in (*a*). In these determinations, creatine was measured by Jaffe's reaction, which is not very specific and, in particular, is given also by pyruvic acid. Further, phosphopyruvic acid would be expected to be partly precipitated under the conditions (*a*) above. In interpreting the experiments with glutamic acid, it was particularly important to be aware of any possible interference from pyruvic acid, and therefore the maximum extent to which phosphopyruvic acid might contribute to the values obtained for creatine was estimated as follows.

Table 1. *Possible contribution by phosphopyruvate to phosphocreatine values*

(Guinea pig cerebral-cortex slices (1·48 g.) were distributed between three 100 ml. vessels each containing 15 ml. glycylglycine-glucose saline, and shaken in O_2 for 40 min. Determinations by titration with 0·005 M-$Na_2S_2O_3$ are described in the experimental section.)

	Ca-10% ethanol precipitate	Ca-80% ethanol precipitate
I_2 consumed (μequiv./g. tissue)	4·71	9·83
Equivalent phosphopyruvic acid (μmoles/g. tissue)	0·79	1·64
Equivalent 'creatine' (μmoles/g. tissue)	0·07	0·15

Brain-cortex slices were prepared from two guinea pigs and caused to respire for 40 min. in glycylglycine-glucose saline. They were extracted with trichloroacetic acid and the Ca salts fractionated as indicated above. The precipitates were dissolved or suspended in water and the consumption of I_2 determined according to Lohmann & Meyerhof's (1934) method for phosphopyruvic acid. The I_2 may oxidize substances other than phosphopyruvic acid; however, a maximum figure for the phosphopyruvic acid could be given on the basis of the consumption of 6 equiv. I_2 by 1 mole of the acid (Table 1). The extent to which pyruvic acid reacted as creatine in the Jaffe determination was determined by finding the apparent creatine from 0·25, 1 and 5 μmoles of pyruvic acid, before and after the preliminary acid treatment involved in the Jaffe reaction. The treatment was not found to alter the apparent values, which were between 0·064 and 0·094 μmole creatine/μmole pyruvate. The higher value has been used for calculating in Table 1 the possible interference of phosphopyruvate in the phosphocreatine

determinations. As a normal value for phosphocreatine in brain slices is 1·5 μmoles/g., the interference could not be more than 10% and is probably less. There is thus no reason to doubt that the previous characterization of phosphocreatine was adequate.

Acid hydrolysis of phosphates from slices. This also required about 1·5 g. of slices, which were caused to respire, extracted, and fractionated by Ca-ethanol as indicated above. Each precipitate was then made up to 5 ml. in water, 5 ml. 2 N-HCl added, and the mixture, in a stoppered tube, put into a bath at 100°. Specimens (2 ml.) were taken at 0, 7, 15 and 30 min. and their inorganic phosphate determined by Fiske & Subbarow's method (see Hawk *et al.* 1947).

RESULTS

Glucose and oxygen on phosphates

The resynthesis of phosphocreatine which took place in oxygenated salines containing glucose, did not take place in the absence of glucose or oxygen. Values are quoted in Table 2. The inorganic phosphate in absence of glucose or oxygen was higher by an amount about equivalent to the fall in creatine phosphate. Respiration continued in the absence of glucose for much longer than the experiments of Table 2, but at a rate which was initially somewhat less than that in the presence of glucose, and which gradually fell further.

Table 2. *Glucose and oxygen on phosphates*

(Bicarbonate-glucose saline with Ca was used and the gases contained 5% (v/v) CO_2. Experiments were at 37° for 40 min. Phosphates were determined after chemical separation.)

Conditions	Inorganic phosphate (μmoles/g.)	Creatine phosphate (μmoles/g.)
With glucose and O_2	3·82	1·33
No glucose; with O_2	5·02	0·31
With glucose and O_2	3·10	1·45
Glucose and N_2	3·96	0·31

The equilibrium which exists between adenine polyphosphates and creatine phosphate in muscle probably exists also in the brain *in vivo* (Klein & Olsen, 1947). Its existence in homogenates of cerebral tissues has been demonstrated in these laboratories (Narayanaswami, 1952) and would be expected in the present preparations. These accordingly would be expected to contain adenine polyphosphates. Table 3 gives evidence indicating that this is the case. In examining the effects of different agents on the phosphates of slices as described in the following sections, our observations have at present been confined to the inorganic and creatine phosphates. This is because determination of, for example, the adenine polyphosphates by the present methods require more tissue, and also because the inorganic and creatine phosphates show the greatest susceptibility to applied agents *in vivo* (Stone, Webster & Gurdjian, 1945; Klein & Olsen, 1947).

Glutamate on phosphates

Determination after calcium separation. A major redistribution in the phosphates of brain-cortex slices took place when glutamate was added to media in which they were respiring. Table 4 (Exp. 1) shows the large fall in phosphocreatine which occurred when glutamic acid was added to media containing glucose. Glutamine had nearly the same effect. The fall took place even in the small quantity of phosphocreatine which remained in the slices after their metabolism in the absence of added substrate (Exp. 3). In all the experiments of Table 4 relatively little change in inorganic phosphate was associated with the addition of glutamic acid or glutamine.

When glutamate replaced glucose as substrate, changes in both inorganic phosphate and phosphocreatine were found. Table 5 shows this, together with the concomitant changes in respiration. Phosphocreatine fell markedly on replacing glucose by glutamate; inorganic phosphate and also respiration rose. Results derived from groups of experiments on two different occasions are given separately; no significant difference existed in values with glucose, but the variations between the two groups of experiments with glutamate as substrate were real. The most likely point for such difference to arise was in the preparation or extraction of the tissue. The effect of changing substrate is, however, far beyond any such difference.

The values of Tables 4 and 5 were obtained after chemical separation of creatine and inorganic

Table 3. *Acid hydrolysis of phosphate fractions from brain cortex*

(Experimental conditions as Table 1, using 1·4–1·8 g. of guinea pig brain-cortex slices. Hydrolysis with N-HCl at 100°.)

	Ca-10% ethanol ppt. Increased inorganic PO_4 (μmoles/g.) after hydrolysis for			Ca-80% ethanol ppt. Increased inorganic PO_4 (μmoles/g.) after hydrolysis for		
Exp.	7 min.	15 min.	30 min.	7 min.	15 min.	30 min.
1	1·34	1·94	2·0	0·36	0·46	1·04
2	1·92	—	2·55	—	—	—
3	1·47	—	2·53	—	—	—

phosphates. The inorganic phosphate was normally measured as the phosphate insoluble in calcium-10% ethanol by Fiske & Subbarow's method, but the same values were obtained by that of Lowry & Lopez (Table 6). Determination of the phosphate of the calcium-80% ethanol precipitate, by the same methods, showed the separation to have been good. Moreover, added glutamic acid did not interfere with the separation or determinations (Exp. 2, Table 6). The arrangement in this case was as follows. Stern *et al.* (1949) showed slices initially containing about 18 μmoles/g. of glutamic acid, to increase this amount to up to 44 μmoles/g. during respiration in the presence of the acid. Amounts of glutamic acid corresponding to 13, 20 and 45 μmoles/g. respectively were therefore added to the extracts, and the usual separations and determinations of inorganic and creatine phosphates were performed. The findings were independent of added glutamic acid. A similar experiment, not recorded in the tables, showed them to be independent also of added glutamine.

Table 4. *Glutamate and glutamine on phosphates*

(All experiments were aerobic in bicarbonate saline with Ca salts, at 37° for 75 min. Glutamate was the L(+) acid taken to pH 7·2 by NaOH; glutamines *A* and *B* refer to two specimens for one of which we are indebted to Mr D. E. Hughes of Sheffield University. Phosphates were determined after chemical separation.)

Exp.	Substrate	Inorganic phosphate (μmoles/g.)	Creatine phosphate (μmoles/g.)
1	Glucose	3·27	1·35
	Glucose and glutamate (0·02 M)	3·24	0·11
	Glucose and glutamate (0·004 M)	3·15	0·36
2	Glucose	4·17	1·36
	Glucose and glutamine *A* (0·02 M)	4·07	0·40
	Glucose and glutamine *B* (0·02 M)	3·90	0·42
3	None	5·17	0·33
	Glutamate (0·02 M) only	5·31	0·06
	Glutamate (0·004 M) only	5·16	0·07

Table 5. *Effects on phosphates of replacing glucose by glutamates*

(Experiments were aerobic and phosphates determined after chemical separation. The group of six experiments was carried out some 6 months later than the group of 11 or 12.)

No. of experiments	Type of saline; substrate	Respiration (μmoles/g./hr.) Mean	S.D.	Inorganic phosphate (μmoles/g.) Mean	S.D.	Creatine phosphate (μmoles/g.) Mean	S.D.
12	Glycylglycine; glucose (0·013 M)	70·5	5·5	3·40	0·35	1·20	0·17
6	Glycylglycine; glucose (0·013 M)	68	6	3·38	0·31	1·12	0·11
11	Glycylglycine; glutamate (0·02 M)	79	4·4	5·22	0·22	0·32	0·07
6	Glycylglycine; glutamate (0·02 M)	78	7	6·35	0·35	0·16	0·035

Table 6. *Separation of phosphates in trichloroacetic acid extracts of cerebral cortex*

(In Exp. 1, cortex slices had first respired for 40 min. in glycylglycine-glucose saline. In Exp. 2, cortex from the cerebral hemispheres was used 2 min. after death of the animal, and creatine phosphate added to the trichloroacetic acid extract.)

Exp.	Material extracted	Substance determined and method	Ca-10% ethanol ppt. (μmoles/g.)	Ca-80% ethanol ppt. (μmoles/g.)
1	Slices	Phosphate; Lowry-Lopez	3·72	0
	Slices	Phosphate; Fiske-Subbarow	3·85	1·99
2*a*	Cortex	Phosphate; Fiske-Subbarow	7·70	—
	Cortex + 1 mg./ml. glutamic acid	Phosphate: Fiske-Subbarow	7·94	—
	Cortex + 3 mg./ml. glutamic acid	Phosphate: Fiske-Subbarow	7·84	—
2*b*	Cortex	Creatine; Jaffe	—	1·23
	Cortex + 1 mg./ml. glutamic acid	Creatine; Jaffe	—	1·29
	Cortex + 3 mg./ml. glutamic acid	Creatine; Jaffe	—	1·19

Phosphates by differential stability. An earlier paper (McIlwain *et al.* 1951) described the determination of inorganic and creatine phosphates in brain extracts by the different specificities of Lowry & Lopez's (1946) and Fiske & Subbarow's methods. Of these, Lowry & Lopez's determined only in-

organic phosphate, and Fiske & Subbarow's determined inorganic and labile phosphates such as creatine phosphate. Table 6, Exp. 1, shows in an individual extract how this combination of determinations would operate. Table 7 gives values for labile and inorganic phosphates in the extracts, which show that the labile phosphate so determined corresponds to the creatine phosphate by calcium-ethanol fractionation when the method is applied to extracts from slices which had respired with glucose as substrate. However, this was not the case when the method was applied to slices which had respired in solutions containing glutamates. The values for labile phosphates were higher than those for phosphocreatine after calcium separation. This is shown clearly in Table 7 which summarizes some forty experiments carried out at different times in the course of about 18 months in four different media. Both in bicarbonate- and in glycylglycine-glucose salines, the values for inorganic and creatine phosphates show reasonable agreement when measured by the two methods. With glutamate as substrate, all values tended to be higher when determined by differential stability; they were also less regular. As explained above, glutamate or glutamine do not interfere with the calcium separation. It appears likely that a labile phosphate other than creatine phosphate and the adenine phosphates accumulates in cerebral tissues respiring with glutamate.

Calcium and potassium salts on phosphates

The media normally employed in metabolic work with separated tissues contain calcium salts in concentrations approximating to those of blood plasma. The respiration of certain tissues, including cerebral cortex, increases when this calcium is omitted (Dickens & Greville, 1935; Canzanelli *et al.* 1942; Krebs, 1950). In the present experiments the effects of calcium salts have been examined under the three conditions quoted in Table 8, and consistent effects found. In the glucose-containing media, omission of Ca^{++} or lowering of Ca^{++} concentration led to lower creatine phosphate and possibly to higher inorganic phosphate concentrations. In the glutamate medium, the creatine phosphate was already so low that a further change was not detected, but the increase in inorganic phosphate was clear.

When the concentration of potassium salts in balanced salt mixtures is increased, respiration and

Table 7. *Phosphates by* Ca *separation and by differential stability*

(Metabolism was in O_2 at 37° in the salines listed (referred to in experimental section) for 75 min. In bicarbonate salines the values given are means of several experiments, the number of which is given in parenthesis.)

	Inorganic phosphate (μmoles/g.)		Labile phosphate (μmoles/g.)	
Substrate; type of saline	By Ca separation	By stability	By Ca separation	By stability
Glucose; glycylglycine	3·40, 3·38	3·43, 3·06	1·20, 1·12	1·32, 1·4
Glutamate; glycylglycine	5·22, 6·35	6·7, 6·1	0·32, 0·16	0·78, 1·4
Glucose; bicarbonate	3·47 (6)	3·69 (8)	1·42 (6)	1·42 (8)
Glutamate; bicarbonate	5·3 (2)	6·8 (9)	0·07 (2)	1·57 (9)

Table 8. *Calcium and potassium salts on respiration, glycolysis and phosphate of cerebral-cortex slices*

(Experiments were with guinea pig cerebral-cortex slices. Numbers 1 to 3 were in one or two flasks of about 100 ml., each containing approx. 15 ml. saline and about 300 mg. tissue. Number 4 was in flasks of about 60 ml., with 8 ml. saline and about 100 mg. of tissues. Flasks were placed in a thermostat at 37° about 30 min. after death of the animal, and the manometric readings on which respiration rates were based, taken at 5 min. intervals. Exps. 1–3 ran for 40 min. and the rates were constant during this period, after which each flask in turn was sampled. Exp. 4 continued for 120 min.)

Exp.	Type of saline; substrate	K^+ (mM)	Ca^{++} (mM)	Respiration (μmoles/g./hr.)	Inorganic phosphate (μmoles/g.)	Creatine phosphate (μmoles/g.)	Lactic acid (μmoles/g./exp.)
1	Glycylglycine; glucose	6·7	0	107·4	4·4	1·03	28
	Glycylglycine; glucose	6·7	2·85	66·4	—	1·49	20
2	Bicarbonate; glucose	6·2	0	—	3·56	0·54	—
	Bicarbonate; glucose	6·2	0·71	—	3·51	0·68	—
	Bicarbonate; glucose	6·2	2·85	—	3·24	1·22	—
3	Glycylglycine; glutamate	6·7	0	86·6	7·28	<0·15	—
	Glycylglycine; glutamate	6·7	0·71	72·2	6·85	<0·15	—
	Glycylglycine; glutamate	6·7	2·85	62·0	5·60	<0·15	—
4	Glycylglycine; glucose	6·7	2·85	68	4·1	0·93	46
	Glycylglycine; glucose	106·7	2·85	103	5·1	0·45	161

glycolysis increase (Ashford & Dixon, 1935; Dickens & Greville, 1935; Canzanelli *et al.* 1942. Here again (Table 8) these actions are seen to be associated with lowered creatine phosphate.

DISCUSSION

In the present study, substances have been encountered which bring about the following group of changes in brain-cortex slices: decreased content of creatine phosphate, increased inorganic phosphate, increased respiration and increased glycolysis. This group of changes has long been recognized to follow increased activity in muscle, and more recently similar phenomena have been shown in the brain *in vivo*. The technique used in the present series of studies with sections of tissue *in vitro* has, however, enabled an interesting variety of agents to be studied under uniform conditions. The agents now observed to cause these changes have been high K^+ concentration, low Ca^{++} concentration, glutamates and glutamine. Previously (McIlwain & Gore, 1951) 2:4-dinitrophenol and electrical stimulation were shown to cause similar changes.

This group of changes is a specific one which differs from those caused for example by narcotics under the same conditions (Buchel & McIlwain, 1950); many other substances including some convulsants and anticonvulsants (Anguiano & McIlwain, 1951, and unpublished) have been found to be relatively inert, or again, to behave differently. In attempting to understand the control of carbohydrate metabolism in nervous tissues, it is therefore important to seek a basis for the group of changes caused by potassium and calcium salts, glutamate, glutamine, dinitrophenol and electrical stimulation. The following suggestions are made in this connexion:

(1) Increased glucose usage might be the first common factor in the action of these substances, its phosphorylation being at the expense of the energy-rich phosphates, and the increase in inorganic phosphate coming from further metabolism of the intermediary hexose phosphates. Normally, however, increased glucose catabolism leads to esterification of inorganic phosphate and not to its formation.

(2) Activation or inhibition of tissue phosphatases, phosphokinases or comparable systems might be the primary effect. Thus calcium and potassium salts have opposing effects on adenosinetriphosphatase of brain (Gore, 1951). Evidence against this view in the case of 2:4-dinitrophenol was given by Case & McIlwain (1951). It has been investigated in the case of K^+ by Lipsett & Crescitelli (1950).

(3) Processes best described in terms of the functional activity of the tissue, might be primarily involved. This appears the most suitable explanation in the case of electrical stimulation (McIlwain & Gore, 1951; McIlwain, 1951*a*). Here the chemical events *in vitro* closely parallel those following stimulation *in vivo*, when clear evidence is seen of increased activity in the central nervous system. The increased respiration and glycolysis then appeared to follow from increased concentrations of inorganic phosphate and phosphate acceptors resulting from utilization of energy-rich phosphates. At this point, the mode of action of the dinitrophenol and of electrical stimulation became similar (McIlwain & Gore, 1951). With respect to Ca^{++} and K^+ salts, glutamate and glutamine, it is significant that these are all normal constituents of nervous tissues or their environment. Although it is not necessary that a common mechanism should exist for the effects of all these agents, the following reasons can be seen for their action paralleling that of electrical stimulation.

Potassium and calcium salts. The increased respiration and glycolysis which potassium salts induce are relatively specific to nervous tissues, and are not caused in testis, kidney, yolk sac and tumour tissues (Dickens & Greville, 1935), nor in chick embryo (Needham, Nowinski, Dixon & Cook, 1937). Movement of potassium salts is associated with the passage of nerve impulses, and one suggestion of how chemically derived energy might be applied in supporting the activity of the tissue is that it may perform osmotic work in maintaining the differential ion concentrations normal to the tissue. The K^+ concentration in the tissue is normally about 0·09 M (see, for example, Hoagland & Stone, 1948) and in surrounding fluids about 0·005 M. The external concentration of K^+ which affected phosphates, glycolysis and respiration was 0·03–0·1 M. Increased intake of K^+ and also of water probably occur in nervous tissues in solutions high in K^+ (Fenn, 1940). If the activities of the tissues were directed to maintaining a ratio of 20:1 in K^+ between inside and outside its cells, the added K^+ would greatly increase the expenditure of energy necessary for such maintenance. This is undoubtedly an over-simplification of events, which also involve Na^+ and Ca^{++}. It gives an immediate analogy between the effects of high K^+ concentration and of electrical stimulation. It is deliberately presented as a contrast to the suggestions of Lipsett & Crescitelli (1950) without at present discussing the merits of the two views.

Antagonism between the effects of calcium and potassium salts is an extremely common physiological phenomenon (see Heilbrunn, 1947) and it is therefore not surprising that in the present experiments the effects of diminishing Ca^{++} concentration should be similar to those of increasing K^+ concentration. In particular the antagonism has been shown to hold with respect to metabolic character-

istics of mammalian brain (Dickens & Greville, 1935; Canzanelli *et al.* 1942) and to the electrical behaviour of separated portions of frog brain (Libet & Gerard, 1939). Considering specific ways in which lack of Ca^{++} may lead to increased utilization of energy-rich compounds, lack of Ca^{++} has been observed to lead to spontaneous discharge in nervous tissues (see Fenn, 1940; Monnier & Chevalier, 1942). The extent to which this can manifest itself in the present preparations is not known, but electrical activity occurs in isolated fragments of certain parts of the frog brain (Libet & Gerard, 1939). Further, calcium salts are observed to aid the 'sealing' of damaged cells, including those of nervous tissues (Heilbrunn, 1947). Damage to cells is inevitable in the present preparations, and by minimizing its effects calcium salts may reduce the amount of osmotic work which the cells are required to perform, and thus reduce also their respiration and glycolysis.

Glutamic acid. This is assimilated by brain slices against a concentration gradient (Stern *et al.* 1949) and presumably with the expenditure of energy, so that the increased respiration brought about by its addition might depend on its active assimilation. The acid was also found to have additional specific effects on the phosphates, which require further study, and which resulted in higher concentrations of inorganic phosphates than those found to follow any of the other agents listed above. It is possible that the labile phosphate accumulating under these conditions may be the γ-glutamyl phosphate suggested but not demonstrated by Elliott (1951) as an intermediate in glutamine synthesis in brain. Higher K^+ concentration is also found in the slices (Krebs & Eggleston, 1949; Krebs, Eggleston & Terner, 1951). Supplying the tissue with glutamic acid may therefore provide it with the means of carrying out more osmotic work and chemical synthesis. There is evidence that, anaerobically, glutamic acid limits glycolysis by depleting the tissue of adenine polyphosphates, for the glycolysis, so limited, is restored by added adenosinetriphosphate (Stern *et al.* 1949). When such depletion occurs aerobically, as in the present experiments, it can be understood on grounds referred to above, that glycolysis and respiration should be stimulated. This was observed by Weil-Malherbe (1938) and occurred during the present experiments.

These observations lead one to expect that the substances studied here would change the response of cerebral tissues to electrical stimulation. This has been found to be the case with glutamic acid (McIlwain, 1951*b*) and ionic constituents (Gore & McIlwain, 1952).

SUMMARY

1. The content of certain phosphates has been investigated in slices of cerebral cortex which were metabolizing in balanced salines containing sodium, potassium, magnesium and calcium salts with chlorides, sulphates, phosphates and sometimes bicarbonates and glycylglycine.

2. When oxygen and glucose were added to the salines, such slices were found to contain creatine phosphate and adenine polyphosphates and their inorganic phosphate was about 3·5 μmoles/g. fresh weight. In the absence of glucose or oxygen, the creatine phosphate fell and the inorganic phosphate rose.

3. The further addition of glutamic acid salts or of glutamine caused the phosphocreatine of the slices to fall, whether or not glucose was also present.

4. When glutamic acid salts replaced glucose as the added substrate, the inorganic phosphate of the slices became greater, and their phosphocreatine much less. A labile phosphate other than phosphocreatine and the adenine phosphates was found in the slices.

5. Lowering the concentration of calcium ion in the salines or increasing that of potassium ion, led to lowered phosphocreatine in the slices and sometimes to increased inorganic phosphates.

6. The various changes in salines described above, were associated with changes also in respiration and glycolysis in the slices. Reasons are given for considering these changes in many cases to be secondary to the changes in phosphates, and to represent a pattern of changes similar to that seen in the intact central nervous system in different states of activity.

I am greatly indebted to Mr J. Cheshire for assistance during some of these experiments, and to Dr G. Anguiano and Dr M. B. R. Gore for permission to quote certain of the results concerning glutamate and potassium salts.

REFERENCES

Anguiano, G. & McIlwain, H. (1951). *Brit. J. Pharmacol.* **6**, 448.
Ashford, C. A. & Dixon, K. C. (1935). *Biochem. J.* **29**, 157.
Buchel, L. & McIlwain, H. (1950). *Brit. J. Pharmacol.* **5**, 465.
Canzanelli, A., Rogers, G. & Rapport, D. (1942). *Amer. J. Physiol.* **135**, 309.
Case, E. M. & McIlwain, H. (1951). *Biochem. J.* **48**, 1.
Dickens, F. & Greville, G. D. (1935). *Biochem. J.* **29**, 1468.
Elliott, W. H. (1951). *Biochem. J.* **49**, 106.
Fenn, W. O. (1940). *Physiol. Rev.* **20**, 377.
Gore, M. B. R. (1951). *Biochem. J.* **50**, 18.
Gore, M. B. R. & McIlwain, H. (1952). *J. Physiol.* (in the Press).

Hawk, P. B., Oser, B. L. & Summerson, W. H. (1947). *Practical Physiological Chemistry*. London: Churchill.
Heilbrunn, L. V. (1947). *An Outline of General Physiology*. Philadelphia: Saunders.
Hoagland, H. & Stone, D. (1948). *Amer. J. Physiol.* **152**, 423.
Klein, J. R. & Olsen, N. S. (1947). *J. biol. Chem.* **167**, 747.
Krebs, H. A. (1950). *Biochim. biophys. Acta, Amst.*, **4**, 249.
Krebs, H. A. & Eggleston, L. V. (1949). *Biochem. J.* **44**, vii.
Krebs, H. A., Eggleston, L. V. & Terner, C. (1951). *Biochem. J.* **48**, 530.
Libet, B. & Gerard, R. W. (1939). *J. Neurophysiol.* **2**, 153.
Lipsett, M. N. & Crescitelli, F. (1950). *Arch. Biochem.* **28**, 329.
Lohmann, K. & Meyerhof, O. (1934). *Biochem. Z.* **273**, 60.
Lowry, O. H. & Lopez, J. A. (1946). *J. biol. Chem.* **162**, 421.
McIlwain, H. (1951*a*). *Biochem. J.* **49**, 382.
McIlwain, H. (1951*b*). *J. ment. Sci.* **97**, 674.
McIlwain, H., Buchel, L. & Cheshire, J. D. (1951). *Biochem. J.* **48**, 12.
McIlwain, H. & Gore, M. B. R. (1951). *Biochem. J.* **50**, 24.
Monnier, A. M. & Chevalier, R. (1942). *C.R. Soc. Biol., Paris*, **136**, 470.
Narayanaswami, A. (1952). *Biochem. J.* **52**, 295.
Needham, J., Nowinski, W. W., Dixon, K. C. & Cook, R. P. (1937). *Biochem. J.* **31**, 1185.
Stern, J. R., Eggleston, L. V., Hems, R. & Krebs, H. A. (1949). *Biochem. J.* **44**, 410.
Stone, W. E., Webster, J. E. & Gurdjian, E. S. (1945). *J. Neurophysiol.* **8**, 233.
Weil-Malherbe, H. (1938). *Biochem. J.* **32**, 2257.

Creatine Phosphokinase in Mammalian Brain

By A. NARAYANASWAMI*
Biochemical Laboratories, Institute of Psychiatry (British Postgraduate Medical Federation, University of London), Maudsley Hospital, S.E. 5

(*Received* 21 *January* 1952)

Different levels of activity in the central nervous system are associated with different contents of energy-rich phosphates and of inorganic phosphate in cerebral tissue (for references and a discussion see McIlwain, 1950). For this reason it was decided to study enzyme systems in cerebral tissue which bring about changes in these substances. Some properties of the adenosinetriphosphatases of brain have already been investigated in these laboratories (Gore, 1951). The present study deals with the systems which lead to the transfer of phosphate from phosphocreatine to adenylic acid or adenosinediphosphate. Lohmann (1934) showed that cold water extracts of minced muscle contain an enzyme which brings about the degradation of phosphocreatine. The phosphate group of phosphocreatine was transferred to adenylic acid resulting in the formation of 'adenylpyrophosphate' (adenosinetriphosphate). Lehmann (1935, 1936) found that this reaction was made up of two reversible bimolecular reactions namely,

Adenosinetriphosphate + creatine (ATP) $\rightleftharpoons$ adenosinediphosphate + phosphocreatine, (ADP)

Adenosinediphosphate + creatine $\rightleftharpoons$ adenosinemonophosphate + phosphocreatine.

Banga (1943) considered that two distinct enzymes were present in muscle, one catalysing the reaction between ATP and creatine and the other catalysing the reaction between ADP and creatine. She obtained the first enzyme in a highly purified state.

The importance of the creatine phosphokinase system in maintaining the level of ATP in muscle has been recognized for some time, but its occurrence in brain does not seem to have been investigated. The present paper reports the presence of this enzyme system in brain and describes some of its properties. Effects on this enzyme system of a few substances structurally related to its substrates, and of some drugs which act on the central nervous system, have also been studied.

EXPERIMENTAL

Preparation of sodium phosphocreatine. This was carried out according to the method of Ennor & Stocken (1948). The product contained 8·05% phosphocreatine P and was practically free from inorganic phosphate.

Adenosinediphosphate. This was prepared as the Ba salt from commercial ATP by heating in the presence of $MgCl_2$ as described by Bielschowsky (1950). The adenylic acid used was a commercial preparation obtained from L. Light and Co. Ltd.

Determination of phosphokinase activity. The reaction mixture usually contained 1 ml. 0·015–0·016 M-sodium phosphocreatine, 1 ml. 0·018 M-adenylic acid adjusted to pH 7 with NaOH, 2·5 ml. of 0·1 M-glycylglycine buffer at pH 7, and 0·5 ml. enzyme preparation containing 0·01 M-$MgSO_4$. The solutions were mixed in that order and were kept cool in ice. This gave phosphocreatine at 3·0–3·2 mM, similar to its concentration in normal brain, and adenylic

* British Council Scholar.

acid about 10% in excess of that required for interaction with the phosphocreatine. The mixture was then incubated in a thermostatically controlled water bath at 37°, and 2 ml. samples were pipetted out at suitable intervals (usually 20 min. and 1 hr.) into 2 ml. 10% (w/v) trichloroacetic acid at 0°. The reaction mixture was kept cool in ice during intervals of pipetting. Blanks were run with each series of experiments, omitting the enzyme preparation.

Determination of phosphocreatine. This was done according to the method of Stone (1943) with slight modifications. The reaction mixture after addition to trichloroacetic acid was allowed to stand for 15 min., centrifuged while being kept surrounded with ice and a 3 ml. sample of the supernatant removed for analysis. To this was added a drop of 0·1% phenolphthalein and just enough finely ground $Ca(OH)_2$ to turn it a pale pink. The $Ca(OH)_2$ was rubbed in the tube with a glass rod, so that no undissolved particles remained, and the glass rod was washed down with a few drops of water. Ethanol which had been adjusted to a faintly alkaline reaction with NaOH was then added to a concentration of 10% (v/v), the solution was well mixed by swirling between the palms and allowed to stand in ice for 15 min. After centrifuging and decanting the supernatant, the precipitate was washed with 1 ml. of a solution of 5% calcium trichloroacetate in 10% (v/v) ethanol, prepared by carrying out a blank precipitation. The washings were added to the centrifugate, the volume made up to 10 ml. after addition of a drop of 5N-HCl and the phosphocreatine in a 2 ml. portion was determined by the method of Fiske & Subbarow as described by Hawk, Oser & Summerson (1947). The final volume for colour development was 10 ml. and an interval of 40 min. was allowed after addition of the reagents. The readings were taken in a Spekker absorptiometer.

Preparation of brain homogenates and extracts

Guinea pig brain was used in most of the experiments, though rat brain was found to be equally active; mention is made of the fact when rat brain has been used in any particular experiment. The guinea pig or rat was stunned by a blow on the neck, decapitated and the brain rapidly removed.

Homogenates. The brain was weighed quickly and transferred to a glass test-tube homogenizer containing a known volume of 0·9% NaCl. It was then homogenized and after this stage the brain preparation was kept throughout at 0° and dilutions were carried out with ice-cold 0·9% NaCl.

Extracts. In later experiments it was found advantageous to use the supernatant obtained on centrifuging the homogenate. In these experiments the homogenate was prepared using 2 ml. 0·9% NaCl/g. brain tissue, the homogenate was allowed to stand at 0° for 5 min. and then centrifuged in the cold. After pouring off the supernatant, the residue was suspended in the same volume of 0·9% NaCl as before and again homogenized. The centrifuging was repeated and the residue was homogenized a third time, using the same volume of 0·9% NaCl. The supernatants were combined and diluted to give an extract equivalent to 100 mg. of brain tissue/ml. This extract contained 42·4% of the N originally present in the tissue. All extracts mentioned in the tables were made in this way unless otherwise stated.

Table 1. *Splitting of phosphocreatine by brain homogenates*

(Total vol. 7 ml.; 0·033M-glycylglycine at pH 7.)

Substrates added	Final dilution of homogenate (mg. tissue/ml.)	Inorganic phosphate (μg. P/ml. reaction mixture) 0 min.	20 min.	1 hr.	Phosphocreatine (μg. P/ml. reaction mixture) 0 min.	20 min.	1 hr.
None	28·6	13·3	14·7	21·3	0·7	0	0
Phosphocreatine	0	21·3	20·7	22·7	89·3	91·7	91·3
Phosphocreatine	28·6	45·0	90·0	96·0	89·3	46·0	34·3
None	9·5	1·0	0·33	1·33	0	0	0
Phosphocreatine	9·5	38·0	31·0	33·7	84·7	89·3	91·7
Phosphocreatine + adenylic acid	9·5	27·3	58·3	83·7	90·3	22·3	0
	2·4	13·7	24·7	41·0	98·3	83·3	41·3
	1·2	12·7	15·3	25·0	98·3	90·0	70·0

RESULTS

Occurrence of creatine phosphokinase in brain. Brain homogenates at a final dilution of 1 g. tissue/35 ml. were found to split phosphocreatine, but this reaction stopped when the dilution of the homogenate was increased to 1 g./105 ml. (Table 1). However, at the latter dilution, the addition of adenosine-5-phosphoric acid brought about the splitting of phosphocreatine at an even faster rate than with higher concentrations of homogenate in the absence of adenylic acid. Thus the splitting of phosphocreatine takes place largely according to the reaction of Lohmann (1934) and not by a simple phosphatase type of enzyme.

Solubility of brain phosphokinase and its distribution in the homogenate. Table 2 shows that the phosphokinase activity of brain could be practically completely extracted by either 0·9% sodium chloride or water. All the activity did not pass into solution on a single extraction with either sodium chloride solution or water, but after the second extraction there was only a negligible amount of activity remaining in the solid residue. The homogenate was allowed to stand in the cold for 5 min. after each extraction and vigorously shaken for 1 min. before centrifuging. The values for inorganic phosphate suggested that there was a comparatively greater ATPase activity in the residue, especially

Table 2. *Solubility of the enzyme in 0·9 % sodium chloride and in water*

(*A*: brain homogenized with 4 parts 0·9 % NaCl, centrifuged and residue stirred up (in homogenizer) with 4 parts of the saline and centrifuged, stirred up again with 4 parts saline as before and centrifuged, the three extracts and the final residue tested for activity separately at final dilutions corresponding to 5·7 mg. tissue/ml. *B*: extraction carried out with glass-distilled water in place of 0·9 % NaCl; same procedure as *A*, final dilution corresponding to 4·8 mg. tissue/ml., 3 mM-$MgSO_4$.)

	Inorganic phosphate (μg. P/ml.)			Phosphocreatine (μg. P/ml.)		
	0 min.	1 hr.	Increase	0 min.	1 hr.	Decrease
			A. Saline extraction			
First extract	15·3	38·0	22·7	94·3	0	94·3
Second extract	18·7	27·3	8·6	92·3	74·0	18·3
Third extract	13·7	19·0	5·3	98·3	93·7	4·6
Residue	16·0	31·7	15·7	97·9	84·7	13·0
			B. Water extraction			
First extract	24·0	61·3	37·3	88·7	0	88·7
Second extract	15·3	36·3	21·0	91·7	19·7	72·0
Third extract	14·3	21·0	6·7	95·7	93·7	2·0
Residue	20·7	24·7	4·0	96·0	92·0	4·0

when extraction is carried out with 0·9 % sodium chloride. More of the phosphokinase activity is extracted by sodium chloride solution in the first extraction than by distilled water.

Table 3. *Activating effect of magnesium ions*

(Dialysed (2 hr.) extract at dilution of 2 mg./ml.; 90·5 μg. phosphocreatine P/ml.)

Concentration of $MgSO_4$ in reaction mixture (mM)	Phosphocreatine split in 20 min. (μg. P/ml.)	Phosphocreatine split in 1 hr. (μg. P/ml.)
0	0·6	18·1
0·5	50·0	85·2
1	58·1	90·5
2	57·2	90·5
5	37·1	90·5

Activating effect of magnesium ions. Dialysis against 0·9 % sodium chloride for 6 hr. completely abolished the phosphokinase activity of a brain extract. The activity was reduced to 86 % after 2 hr. dialysis and to 34 % after 4 hr. dialysis. The tests were carried out at a final dilution of 5 mg./ml. (calculated on the weight of brain tissue from which the extract was prepared). The activity of the dialysed extract was not restored by the addition of boiled extract to a final dilution of 10 mg./ml., but such an addition to the undialysed extract enhanced its activity considerably. The activating effect of boiled extracts was due to its content of magnesium ions. At dilutions as low as 2 mg./ml. the activity even of undialysed extracts was found to be considerably enhanced by the addition of magnesium ions. The optimal concentration of magnesium ions added as magnesium sulphate was found to be 1–2 mM (Table 3). The rate of reaction was perceptibly slower at higher concentrations of magnesium sulphate, as for instance 5 mM. Both calcium ions (as calcium chloride) and manganese ions (as manganous sulphate) restored the activity of dialysed extracts, but were not nearly as effective as magnesium ions (Table 4). Potassium ions added as potassium chloride were without any activating effect on the enzyme.

Table 4. *Activating effect of calcium, manganese and potassium ions*

(Dialysed (2 hr.) extracts at final dilution of 2 mg./ml.)

Activator added	Concentration of activator (mM)	Phosphocreatine (μg. P/ml.) Present initially	Split in 20 min.	Split in 1 hr.
None	—	101·7	0·8	20·3
Magnesium sulphate	1	101·7	74·7	100·0
Potassium chloride	10	101·7	4·6	24·4
Potassium chloride	1	101·7	0	19·7
Calcium chloride	5	90·5	15·7	90·5
Calcium chloride	2	90·5	25·5	84·5
Calcium chloride	1	90·5	21·1	—
Manganous sulphate	1	97·0	36·7	93·9
Manganous sulphate	0·2	97·0	37·9	77·5

pH *optimum of the forward reaction*. The change: phosphocreatine + adenylic acid → creatine + ADP was optimally catalysed at a pH of 7, although decreasing the pH to 5·9 altered the reaction rate very little (Table 5). The rate of reaction decreased more steeply with increasing pH and was practically abolished at pH 8·9. Below pH 5·9 the splitting of phosphocreatine that took place was not dependent on the enzyme at all, the rate of splitting being almost the same in the absence of the enzyme.

Table 5. pH *Optimum of brain phosphokinase*

(Dilution of extract, 2 mg./ml.; 5 mM-$MgSO_4$; 92 μg. phosphocreatine P/ml. originally present.)

pH	Buffer	Phosphocreatine split (μg. P/ml.) 20 min.	Phosphocreatine split (μg. P/ml.) 1 hr.	Phosphocreatine split in absence of enzyme (μg. P/ml.) 1 hr.
4·1	0·1 M-acetate, acetic acid	45	75	73·2
5·0	0·1 M-acetate, acetic acid	14	36·3	31·9
5·9	0·1 M-acetate, acetic acid	55	89·3	6·9
7·0	0·1 M-glycylglycine	60	89·3	0
8·2	0·1 M-glycine-sodium hydroxide	14·7	51·3	0·7
8·9	0·1 M-glycine-sodium hydroxide	6·3	6·0	0·3

Adenosinediphosphate as phosphate acceptor. When ADP was used as the phosphate acceptor in place of adenylic acid, the reaction was not so fast as with adenylic acid and was still incomplete at the end of 1 hr. (Table 6). The pH optimum was, however, within the same range. The evidence for the functioning of ADP as an alternative phosphate acceptor is, however, incomplete, since the possibility remains that an enzyme similar to myokinase might produce adenylic acid from the added ADP.

Table 6. *Enzyme activity with ADP as phosphate acceptor*

(Buffers as in Table 5; 5 mM-$MgSO_4$; 2·83 mM-ADP; dilution of extract 2 mg./ml.; 82·7 μg. phosphocreatine P/ml.)

pH	Phosphocreatine split (μg. P/ml.) 20 min.	Phosphocreatine split (μg. P/ml.) 1 hr.	Phosphocreatine split in absence of enzyme (μg. P/ml.) 1 hr.
4·1	38·7	62·7	65·1
5·0	17·7	43·4	28·3
5·9	40·4	51·4	6·1
7·0	47·0	56·4	0
8·2	22·7	38·7	0·6
8·9	6·0	12·4	0·3

Stability of the enzyme. The activity of the enzyme did not decrease markedly on storage in the cold for periods of up to 8 days. The enzyme stored for 8 days split added phosphocreatine completely in 1 hr. at the usual concentration, though in the first 20 min. it split only 64 % of the phosphocreatine as compared to 85 % by the fresh preparation.

Synthesis of phosphocreatine by brain phosphokinase

The creatine phosphokinase of muscle is known to catalyse the synthesis of phosphocreatine from creatine and ATP (Lehmann, 1935; Banga, 1943). With preparations from brain it was found that a much higher concentration of the enzyme (20 mg. tissue/ml.) was required for synthetic activity than for the splitting of phosphocreatine (2 mg. tissue/ml.). It is probable that the presence of ATPase in the brain extract limits the speed and extent of phosphocreatine synthesis, and this might explain the lesser synthetic activity at higher concentrations of brain extract. The pH range favouring this reaction (Table 7) in the presence of enzyme preparations from brain, seems to be the same as for muscle phosphokinase (Banga, 1943). The reaction proceeds at a measurable rate only above pH 8·2. An equilibrium is reached in about 40 min. and thereafter no increase in phosphocreatine can be detected. The reaction appears to be slower than with comparable concentrations of an extract from muscle.

Table 7. *Effect of* pH *and enzyme concentration on synthesis of phosphocreatine*

(2·8 mM-ATP; 5·5 mM-creatine; 5 mM-$MgSO_4$; 0·1 M-glycine-sodium hydroxide buffer.)

pH	Dilution of enzyme extract (mg. tissue/ml.)	Phosphocreatine formed (μg. P/ml.) 20 min.	Phosphocreatine formed (μg. P/ml.) 1 hr.
7·0	20	0	0
8·2	20	3·3	0
8·9	20	18·7	14·7
8·9	40	11·7	5·0
8·9	10	8·3	10·3

The effect of structural analogues, drugs of central action and some inhibitory agents

Various drugs known to act on the central nervous system were tested for their effect on the phosphokinase activity of brain extract (Table 8). These included the convulsants caffeine, leptazol, picrotoxin and strychnine, the anticonvulsants

Table 8. *Effect of various substances on enzyme activity*

(Enzyme extract at dilution of 2 mg./ml.; 1·0 mM-$MgSO_4$.)

Exp.	Inhibitor added	Concentration of inhibitor (mM)	Phosphocreatine split (μg. P/ml.) 20 min.	Phosphocreatine split (μg. P/ml.) 1 hr.	Inhibition based on 20 min. values (%)
A	None	—	55·3	61·0	—
	Caffeine	10	56·6	61·0	Nil
	Leptazol	10	56·9	61·0	Nil
	Phenobarbitone	10	53·8	61·0	Nil
	Dial (5:5-diallylbarbituric acid)	10	57·8	61·0	Nil
B	None	—	33·3	82·3	—
	Adenine	2	39·1	82·3	Nil
	Adenosine	10	36·0	82·3	Nil
	Picrotoxin	2	35·7	82·3	Nil
	Mesantoin (3-methyl-5:5-phenylethylhydantoin)	2	30·3	82·3	Nil
	Morphine	1	39·9	82·3	Nil
	Atropine	1	40·8	82·3	Nil
C	None	—	53·1	81·7	—
	Strychnine	1	52·5	81·7	Nil
	Barbitone	10	49·3	81·7	Nil
	Tridione (3:5:5-trimethyl-2:4-oxazolidinedione)	10	50·6	81·7	Nil
	Iodoacetate	1	0	0	100
	Iodoacetate	0·5	—	3·4	—
	Iodoacetate	0·2	24·2	43·4	57
D	None	—	52·4	73·5	—
	Sodium fluoride	50	54·6	53·1	Nil*
	Sodium fluoride	10	37·7	60·0	28
	Sodium fluoride	1	42·7	73·5	18
	2:4-Dinitrophenol	1	43·3	68·5	17
	(+)-Tubocurarine	1	51·8	71·5	Nil

* Fluoride at this concentration was found to interfere with the estimation of phosphocreatine.

phenobarbitone, tridione (3:5:5-trimethyl-2:4-oxazolidinedione) and mesantoin (3-methyl-5:5-phenylethylhydantoin), and the narcotics barbitone, dial (5:5-diallylbarbituric acid) and morphine. None of these drugs, even when present at the high concentrations indicated, had any significant effect on the splitting of phosphocreatine by brain extracts. A few of these drugs were also studied for their effect on the reverse reaction of phosphocreatine synthesis, since this takes place at a different pH and some of these substances might be expected to behave differently with an altered state of ionic equilibrium (Table 9). However, no marked reduction of synthetic activity could be detected with any of the substances examined.

Table 9. *Effect of various substances on phosphocreatine synthesis*

(Dilution of extract, 20 mg./ml.; 0·1 M-carbonate-bicarbonate buffer at pH 8·77; 5·6 mM-creatine; 3·2 mM-ATP.)

Added substance	Concentration (mM)	Phosphocreatine formed (μg. P/ml.) 20 min.	Phosphocreatine formed (μg. P/ml.) 40 min.
None	—	10·3	13·1
2:4-Dinitrophenol	0·1	10·0	13·4
Dial (5:5-diallyl-barbituric acid)	10	6·7	13·0
Phenobarbitone	10	6·3	11·9
Leptazol	10	8·3	10·8
Caffeine	10	6·9	9·9
Tridione (3:5:5-trimethyl-2:4-oxazolidinedione)	10	10·1	11·7

Adenine and adenosine, which are structurally related to the substrates, were also found to be without any effect on enzyme activity. Of the miscellaneous substances tested, namely fluoride, iodoacetate, atropine, 2:4-dinitrophenol and (+)-tubocurarine, only iodoacetate inhibited enzyme activity significantly, 100 % at 1 mM and 57 % at 0·2 mM, while the inhibition caused by fluoride (28 % at 10 mM) was not as high as the activating effect of magnesium ions might lead one to expect.

Manometric measurement of phosphokinase activity

In an attempt to follow the time course of the phosphokinase reaction in greater detail than was practicable by the technique of colorimetric estimation of phosphocreatine remaining after varying periods of incubation, several experiments were carried out to test the feasibility of a manometric method of following this reaction. The reaction mixture in the main compartment of the manometric vessel contained, in a volume of 2·5 ml., 4–10 μmoles sodium phosphocreatine, 4·5–12 μmoles adenylic acid, and $NaHCO_3$ to give

a final concentration of 5·89 mM. The enzyme preparation with 0·03 M-$MgSO_4$ in 0·5 ml., was in the side arm. The gas phase was N_2 containing 5 % (v/v) CO_2. The enzyme solution was tipped into the main compartment when the manometer readings were steady, and readings were taken at 5 min. intervals. At the end of 1 hr. the phosphocreatine remaining in the solution was estimated by the usual method. It was found that the reaction proceeded to completion only with relatively high concentrations of enzyme (10 mg. tissue/ml.); at these concentrations the ratio μmoles CO_2 absorbed to μmoles phosphocreatine split approximated to unity, as would be expected if the creatine liberated by the reaction absorbed 1 equivalent of CO_2. However, at lower concentrations of enzyme, the reaction did not proceed to completion and the ratio of CO_2 absorbed to phosphocreatine split decreased to nearly 0·5. Reactions other than that of the phosphokinase were found to be taking place with the higher tissue concentrations, as inorganic phosphate was formed. The method was not examined further.

DISCUSSION

It is evident from this study that phosphocreatine is not degraded in brain by a simple hydrolytic reaction, but that the energy-rich phosphate bond is transferred to a phosphate acceptor, adenylic acid or ADP, so that the stored energy in phosphocreatine largely remains available. In muscle, phosphocreatine acts as a reservoir of energy-rich phosphate bonds. The enzyme system described here might prove to be responsible for the first step in the utilization of phosphocreatine for the functional activity of the central nervous system.

Under optimum conditions of pH and concentration of magnesium ions the extract from 2 mg. brain can bring about the splitting of 2·5 μmoles of phosphocreatine in 20 min. This corresponds to 3750 μmoles/g./hr. Dawson & Richter (1950) found that electrical stimulation *in vivo* produced a 50 % fall in the phosphocreatine content of the brain of rats in 1 sec. At the optimal rate we have found that brain phosphokinase could split 1·04 μmoles of phosphocreatine/g. tissue/sec. As rat brain contains 3·16 μmoles/g., this is of the order reported by Dawson & Richter. We have also observed a slowing of the reaction after the first 20 min. and it is possible that the rate of phosphocreatine splitting might be even greater during the first few seconds. Using the values given by Klein & Olsen (1947) for the glucose and lactic acid content of the brains of cats in their normal condition and after a 10 sec. convulsive period, McIlwain, Anguiano & Cheshire (1951) have estimated possible maximal rates for the metabolic utilization of glucose by brain. They arrived at a figure of -370 μmoles glucose/g./hr. The rate at which the phosphokinase of brain releases high-energy phosphate is thus much greater than is necessary to ensure the phosphorylation and utilization of glucose at the above rate. It is possible that in the intact cell the activity of the phosphokinase is modified by histological and other factors, for instance the concentration of phosphate acceptors. This is an aspect of the enzyme system not dealt with in the present work and deferred for further study.

It is of interest to compare phosphokinase activity with the activities of other enzyme systems present in brain and concerned with the degradation or transfer of energy-rich phosphate groups. Gore (1951) has shown that under optimum conditions the ATPase activity of guinea pig brain is as high as 10·2–13·6 μmoles P/mg./hr. This is on the basis of dry weight of tissue and is equivalent to 1600–2176 μmoles P/g./hr. for wet tissue, and thus is of the same order of activity as observed for phosphokinase. The values reported for hexokinase activity, on the other hand, are very much lower. Meyerhof & Wilson (1948) found that on incubation with a 1:5·5 brain extract, the decrease in glucose was 1·2 μmoles/12 min. This leads to a value of 66 μmoles P transferred/g./hr. Thus phosphokinase in brain could form ATP at a considerably faster rate than would be required for the full activity of hexokinase.

Convulsants and anticonvulsants are known to lead to changes in energy-rich phosphates of the brain *in vivo* (Stone, Webster & Gurdjian, 1945; Klein & Olsen, 1947). There are good reasons for thinking that these changes are secondary to changed functional activities in the central nervous system, but they could be achieved by a purely biochemical mechanism, such as a direct acceleration or inhibition of the phosphokinase system. No indication of such an effect was, however, found.

It is evident also that 2:4-dinitrophenol, which can markedly affect the phosphates in cerebral tissue (McIlwain & Gore, 1951), does not act on the creatine phosphokinase system. The relatively slight inhibition caused by fluoride ions at a concentration at which they might be expected to remove a large part of the magnesium ions from the system is interesting and can probably be explained by the strong affinity of the enzyme for binding magnesium ions. Iodoacetate, the most effective inhibitor found, inhibits respiration of brain slices at comparable concentrations when glucose is the substrate (Krebs, 1931) and it inhibits also a number of enzymes whose activity depends on thiol groups.

SUMMARY

1. Homogenates of mammalian brain have been found to split phosphocreatine in the presence of adenylic acid. The phosphokinase activity of brain homogenates can be recovered in the supernatant on centrifuging them.

2. Magnesium ions at 1–2 mM are necessary for full activity of the dialysed enzyme preparations; manganese and calcium ions are also effective as

activators though less so than magnesium. Potassium ions are inactive in this respect.

3. The pH optimum of the enzyme for the forward reaction, i.e. the splitting of phosphocreatine in the presence of adenylic acid lies in the range of 5·9–7; phosphocreatine is split optimally at the same pH range when adenosinediphosphate is used as the phosphate acceptor.

4. Preparations from brain also catalyse the synthesis of phosphocreatine from creatine and adenosinetriphosphate above pH 8·2.

5. None of the drugs of central action which were examined had any significant effect on the activity of the enzyme. Iodoacetate inhibited it completely above a concentration of 0·5 mM and fluoride to a much less marked degree.

I wish to thank Dr McIlwain for having suggested the problem and for many helpful discussions.

REFERENCES

Banga, I. (1943). *Stud. Inst. med. Chem. Univ. Szeged, Hung.*, **3**, 59.

Bielschowsky, M. (1950). *Biochem. J.* **47**, 105.

Dawson, R. M. C. & Richter, D. (1950). *Amer. J. Physiol.* **160**, 203.

Ennor, A. H. & Stocken, L. A. (1948). *Biochem. J.* **43**, 190.

Gore, M. B. R. (1951). *Biochem. J.* **50**, 18.

Hawk, P. B., Oser, B. L. & Summerson, W. H. (1947). *Practical Physiological Chemistry*, 12th ed. London: Churchill.

Klein, J. R. & Olsen, N. S. (1947). *J. biol. Chem.* **167**, 747.

Krebs, H. A. (1931). *Biochem. Z.* **234**, 278.

Lehmann, H. (1935). *Biochem. Z.* **281**, 271.

Lehmann, H. (1936). *Biochem. Z.* **286**, 336.

Lohmann, K. (1934). *Biochem. Z.* **271**, 264.

McIlwain, H. (1950). *Brit. med. Bull.* **6**, 301.

McIlwain, H., Anguiano, G. & Cheshire, J. D. (1951). *Biochem. J.* **50**, 12.

McIlwain, H. & Gore, M. B. R. (1951). *Biochem. J.* **50**, 24.

Meyerhof, O. & Wilson, J. R. (1948). *Arch. Biochem.* **19**, 502.

Stone, W. E. (1943). *J. biol. Chem.* **149**, 29.

Stone, W. E., Webster, J. E. & Gurdjian, E. S. (1945). *J. Neurophysiol.* **8**, 233.

Effects of Insulin and of Glycerol *in vitro* on the Incorporation of [*carboxy*-^{14}C]acetate into the Fatty Acids of Lactating Mammary Gland Slices with Special Reference to Species Differences

By JUDITH H. BALMAIN, S. J. FOLLEY and R. F. GLASCOCK
National Institute for Research in Dairying, University of Reading

(*Received* 29 *January* 1952)

Udder slices from lactating ruminants (cow, ewe, goat) have been shown actively to utilize acetate as sole substrate with R.Q. > 1 (Folley & French, 1950), from which it was concluded that udder tissue of the ruminant can synthesize fat from acetate alone. On the other hand, slices of lactating mammary gland from non-ruminants (rat, rabbit) were inert to acetate, which, however, was utilized in the presence of glucose, the R.Q. then being of the same order (> 1) as in glucose alone. Moreover, addition of glucose to the medium increased the amount of acetate used by mammary gland slices from ruminants (sheep) and the R.Q. was increased. This was taken to indicate that glucose increases the utilization of acetate for fat synthesis under these conditions.

The effect of insulin *in vitro* on fat synthesis by mammary gland slices has also been studied by respiratory measurements. Addition of insulin to the incubation medium resulted in a marked increase in the R.Q., $-Q_{O_2}$ and acetate uptake of rat mammary gland slices metabolizing mixtures of acetate and glucose, but had no effect on the respiration of the slices in acetate alone, from which it was concluded that in rat mammary tissue insulin enhances fat synthesis from acetate in the presence of glucose, but evokes no fatty acid synthesis in acetate alone (Balmain, French & Folley, 1950; Balmain & Folley, 1951). Sheep udder slices, on the other hand, were inert to insulin both in acetate alone and in acetate plus glucose (Balmain & Folley, 1951). The increase in the R.Q. of mammary gland slices from lactating rats in the presence of insulin has been confirmed by Hills & Stadie (1952).

Glycerol has been found to influence the metabolism of acetate plus glucose by rat mammary gland slices in a way similar to insulin in all respects save that the increase in R.Q. was less marked and, more important, glycerol did not increase $-Q_{\text{glucose}}$ while insulin did. This finding was interpreted as

showing that glycerol increased the rate of fatty acid formation by the slices and thus fat synthesis in the mammary gland might be limited by the amount of glycerol available (Balmain & Folley, 1951).

Since the above conclusions rested largely on R.Q. determinations which are not universally accepted as yielding unequivocal information on the type of reaction occurring within an organ or tissue, it was considered advisable also to investigate these questions by tissue-slice experiments using isotopically labelled acetate. The first of such experiments, in which mammary gland slices from lactating rats were incubated with glucose and [*carboxy*-^{14}C]acetate, the water of the medium being labelled with tritium, showed that the fatty acids synthesized by the slices *in vitro* contained appreciable amounts of tritium and ^{14}C and that the content of both isotopes was increased in presence of insulin (Balmain, Folley & Glascock, 1951). The results of the present, more extensive, experiments, which have been reported in preliminary form elsewhere (Balmain, Folley & Glascock, 1952), confirm the above-mentioned deductions made from respiratory and acid-change measurements in every particular.

METHODS

Experimental animals: incubation of slices. The animals used were primiparous Norway rats (seven to eight young each) killed by dislocation of the neck after 13–15 days of lactation, and three primiparous lactating ewes (one lamb each) shot through the brain after 23–27 days of lactation. In the case of the ewes, each udder, immediately after removal, was dissected into its two constituent glands and separate experiments set up with tissue from each half, making, as in the case of the rats, six experiments in all.

Mammary tissue was sliced with a Stadie & Riggs (1944) microtome. Pooled abdominal mammary tissue from two animals was used for each experiment with rats, batches of slices weighing 2 g. being shaken for 3 hr. at 37° in 100 ml. Warburg flasks containing 20 ml. Krebs bicarbonate saline, the gas phase being 95% O_2 and 5% CO_2(v/v). The medium contained 0·02 M-sodium acetate or 0·02 M-sodium acetate + 0·3% glucose. The acetate was labelled with $CH_3{}^{14}COONa$, the total radioactivity in each 20 ml. medium being 1·0 μc. In each experiment four flasks were set up containing acetate alone and four containing acetate + glucose. In one flask of each of these two series of four the tissue was killed at the outset by the addition of HCl to a concn. of 0·3 N. Crystalline insulin (Novo), free from glycogenolytic factor, was added to the medium in one of the remaining three flasks to a final concentration of 0·05 mg. (approx. 1 i.u.)/ml. and glycerol (final concn. 0·2%) to the medium in another. No addition was made to the fourth flask. The flasks were attached to Warburg manometers so that the steady rise in pressure, indicative of the high R.Q. exhibited by rat mammary gland slices under these conditions, and the enhanced rate of pressure rise due to the addition of insulin or glycerol, could serve as convenient indicators of the progress of the experiment.

The experiments on sheep tissue (3 g. batches of slices) were set up similarly except that no manometric observations were made; the incubations (3 hr.) were carried out in ordinary conical flasks each with a rubber bung carrying a glass tube, dipping under the surface of the medium, through which the gas mixture was bubbled (with constant shaking) throughout the incubation period.

Isolation of total fatty acids. The method used for the isolation of the total fatty acids of the slices was modified from that of Popják & Beeckmans (1950). At the end of the incubation period the tissue was killed by addition of HCl to a concn. of 0·3 N and the contents of each flask homogenized in a Folley & Watson (1948) homogenizer. After neutralization to litmus the mixture was taken practically to dryness under reduced pressure at 40° and the residue refluxed five times for 5 min. with successive 25 ml. lots of ethanol-ether, 3:1 (v/v). The decanted extracts were combined and taken to dryness under reduced pressure at 40° and traces of water removed by distilling 50 ml. benzene from the residue. The residue was refluxed five times for 5 min. with 25 ml. portions of light petroleum (b.p. 40–60°). The combined, filtered extracts were taken to dryness (reduced pressure), and the residue refluxed for 24 hr. with 5 ml. ethanolic KOH (1 vol. 40% KOH:10 vol. absolute ethanol). The solution of soaps was diluted with 10 ml. 50% (v/v) ethanol and extracted with 5 × 50 ml. light petroleum. The solution was then acidified with H_2SO_4 and extracted with 3 × 25 ml. light petroleum. The extract was washed with water until the washings were neutral, 1 drop of phenolphthalein added, and the liquid titrated to a faint permanent pink with $Ca(OH)_2$ solution. The light petroleum was distilled from the solution of Ca salts of the fatty acids and the latter were evaporated to dryness on the steam bath. The Ca salts were not washed with unlabelled acetate since the 'blank' values (Table 1) gave no reason to suspect that they were contaminated with $CH_3{}^{14}COONa$. The samples so obtained were counted with an end-window counter at infinite thickness on 1·0 cm.^{2} disks and the results expressed as counts/min./mg. C by use of an appropriate factor.

Isolation of glycerol. Glycerol was isolated as the tri-*p*-nitrobenzoate from the pooled saponification mixtures after extraction of the fatty acids, there being eight samples (blank, control, insulin, and glycerol each with acetate and glucose + acetate, respectively, as substrates) for each species (rat and sheep). Each solution was made just alkaline to phenolphthalein by addition of KOH and evaporated to dryness on the steam bath. The solid residue was extracted by refluxing with five successive portions of ethyl acetate (25 ml. each) and the solvent distilled off under reduced pressure. The residue was dissolved in 5 ml. pyridine, slightly more than the theoretical amount of *p*-nitrobenzoyl chloride added, the mixture refluxed for 5 min. and cooled for 1 hr. in the refrigerator. Water was added and the glyceryl tri-*p*-nitrobenzoate collected on a sintered-glass filter. The precipitate was washed on the filter with 0·1 N-NaOH and then thoroughly washed with water. It was then washed with 5 ml. 95% ethanol added dropwise and finally given ten washes with 2 ml. portions of acetone, the precipitate being thoroughly stirred each time. The final product dried over P_2O_5 *in vacuo* had m.p. 188° and was assayed for ^{14}C.

RESULTS

Effect of glucose on incorporation of acetate carbon into fatty acids. A comparison of the incorporation of acetate carbon into the total fatty acids of the

mammary gland slices in the presence of acetate and of acetate plus glucose, respectively, is given for both species in Table 1. The results show in the first place that rat mammary gland slices in acetate alone incorporate no acetate carbon into fatty acids under our conditions, while sheep mammary gland slices incorporate appreciable amounts. In the presence of glucose, however, rat tissue is able to utilize considerable amounts of acetate carbon for fatty acid synthesis and, moreover, the incorporation effected by sheep udder slices is increased 2–3 times. The stimulating effect of glucose on fatty acid synthesis from acetate by mammary slices is thus very marked in both rat and sheep mammary tissue.

Effect of insulin on incorporation of acetate carbon into fatty acids. The effect of insulin on the incorporation of acetate carbon into the fatty acids of rat mammary slices is shown in Table 2, and of sheep udder slices in Table 3. It will be seen that insulin evoked no fatty acid synthesis from acetate in rat mammary tissue incubated with acetate alone but, in the presence of glucose, insulin markedly increased the acetate incorporation in all experiments. In sheep udder tissue, irrespective of whether glucose was present, insulin had no detectable effect on fatty acid synthesis from acetate.

Table 1. *Effect of glucose on the incorporation of* ^{14}C *into fatty acids of mammary gland slices*

(The results in this and the following tables are expressed as counts/min./mg. fatty acid C.)

Substrate	Rat mammary tissue Exp. no. 1	2	3	4	5	6	Blank values*
[*carboxy*-^{14}C]acetate	50	43	40	50	58	40	11–58
[*carboxy*-^{14}C]acetate + glucose	5951	3805	5886	3532	3215	1069	0–40
	Sheep mammary tissue Exp. no.† 1L	1R	2L	2R	3L	3R	
[*carboxy*-^{14}C]acetate	3334	3539	2660	2948	4144	4018	29–76
[*carboxy*-^{14}C]acetate + glucose	9533	9911	8975	10249	8086	7441	14–54

* The blank values also apply to the results in the following tables.
† 1L refers to the left udder-half of sheep no. 1, etc.

Table 2. *Effect of insulin on the incorporation of* ^{14}C *into fatty acids of rat mammary gland slices*

Addition	Exp. no. 1	2	3	4	5	6
	Substrate: [*carboxy*-^{14}C]acetate					
Insulin	86	40	97	43	58	58
None	50	43	40	50	58	40
	Substrate: [*carboxy*-^{14}C]acetate + glucose					
Insulin	9061	7567	10847	6383	7132	1991
None	5951	3805	5886	3532	3215	1069

Table 3. *Effect of insulin on the incorporation of* ^{14}C *into fatty acids of sheep udder slices*

Addition	Exp. no.* 1L	1R	2L	2R	3L	3R
	Substrate: [*carboxy*-^{14}C]acetate					
Insulin	4363	3330	2167	2671	3442	3658
None	3334	3539	2660	2948	4144	4018
	Substrate: [*carboxy*-^{14}C]acetate + glucose					
Insulin	9176	10195	9115	7729	7722	7913
None	9533	9911	8975	10249	8068	7441

* 1L refers to the left udder-half of sheep no. 1, etc.

Effect of glycerol on the incorporation of acetate into fatty acids. Tables 4 and 5 show the effects of glycerol on the utilization of acetate carbon for fatty acid synthesis by rat and sheep mammary tissue respectively. In the case of rat tissue in acetate alone, the results indicate that addition of glycerol evoked a slight utilization of acetate carbon for fatty acid synthesis. In glucose plus acetate, however, glycerol almost doubled the utilization of acetate for fatty acid synthesis, the effect being

quite as large as that of insulin. In this connexion it may be noted that in respiratory experiments (Balmain & Folley, 1951) glycerol has given somewhat smaller increases in R.Q. than insulin. In the case of sheep udder tissue in acetate alone, glycerol was effective in markedly increasing the amount of ^{14}C found in the fatty acids, but was ineffective in the presence of glucose.

R.Q. whilst an increase only in turnover rate would not do so; hence the present results taken in conjunction with our previous respiratory measurements leave little doubt that in the present experiments substantial incorporation of ^{14}C into fatty acids is indicative of an increase in the rate of net fatty acid synthesis. The two sets of results, obtained by quite independent techniques, thus rein-

Table 4. *Effect of glycerol on the incorporation of* ^{14}C *into fatty acids of rat mammary gland slices*

	Exp. no.					
Addition	1	2	3	4	5	6
	Substrate: [*carboxy*-^{14}C]acetate					
Glycerol	209	94	151	86	115	158
None	50	43	40	50	58	40
	Substrate: [*carboxy*-^{14}C]acetate + glucose					
Glycerol	10282	8050	10566	6804	7657	2088
None	5951	3805	5886	3532	3215	1069

Table 5. *Effect of glycerol on the incorporation of* ^{14}C *into fatty acids of sheep udder slices*

	Exp. no.*					
Addition	1L	1R	2L	2R	3L	3R
	Substrate: [*carboxy*-^{14}C]acetate					
Glycerol	6880	5040	6534	5731	5753	5148
None	3334	3539	2660	2948	4144	4018
	Substrate: [*carboxy*-^{14}C]acetate + glucose					
Glycerol	8878	9209	8752	8255	7477	7301
None	9533	9911	8975	10249	8068	7441

* 1L refers to the left udder-half of sheep no. 1, etc.

Origin of glyceride glycerol. All samples of glyceryl tri-*p*-nitrobenzoate were virtually non-radioactive, indicating that under our conditions mammary gland slices of either species are unable to incorporate acetate carbon into glycerol.

DISCUSSION

All the deductions, mentioned in the introductory section of this paper, regarding the ability of mammary gland slices to utilize acetate for fatty acid synthesis *in vitro*, and the effects or lack of effects of glucose, glycerol and insulin upon this process in mammary tissue of ruminants and non-ruminants, have been confirmed by the present results; that is, in so far as they show that acetate carbon is incorporated into the fatty acids of lactating mammary tissue under certain defined conditions and that the rate of incorporation is increased in some circumstances by addition of the above-mentioned agents. It is not possible to tell from these results whether in any instance there has been an increased net synthesis of fatty acids or merely an increased turnover rate. However, an increased net synthesis would probably increase the R.Q. whilst an increase only in turnover rate would not do so; hence the present results taken in conjunction with our previous respiratory measurements leave little doubt that in the present experiments substantial incorporation of ^{14}C into fatty acids is indicative of an increase in the rate of net fatty acid synthesis. The two sets of results, obtained by quite independent techniques, thus reinforce and complement one another, and it may be concluded that useful deductions about fat synthesis by mammary gland slices from lactating animals may be drawn from R.Q. measurements with fair confidence.

Certain features of the results are worthy of comment. Addition of glycerol causes a small but definite incorporation of acetate carbon into the fatty acids of rat mammary slices in presence of acetate alone, a substrate towards which the slices are totally inert in absence of glycerol. In view of the marked utilization of acetate for fatty acid synthesis by rat mammary tissue in presence of glucose it would seem reasonable to suppose that rat mammary slices can convert glycerol to glucose or to some metabolite of glucose through which glucose stimulates fatty acid synthesis from acetate. The results show, however, that this conversion must be very slow.

As regards the mechanism of the stimulation of fatty acid synthesis by glucose, it was suggested by Folley & French (1949*b*, 1950) that if, as it is reasonable to suppose, glycerol can be a limiting factor in the synthesis of glycerides by mammary tissue and

hence, at one remove, of fatty acids, the favourable effect of glucose might be due to its breakdown to glycerol. Indeed, French & Popják (1951) later demonstrated the active incorporation of carbon from [^{14}C]glucose into the glycerol of the milk glycerides in the living rabbit. However, since, as shown by the present work, the effect of glycerol on the utilization of acetate for fatty acid synthesis by rat mammary slices is very slight in comparison with that of glucose and, moreover, since both isotope and respiration studies demonstrate that in rat mammary tissue glycerol is effective when glucose is present, this cannot be the complete explanation of the glucose effect, at any rate as regards rat mammary tissue. On the other hand, this theory would suffice to explain our observations on sheep udder slices since in this tissue glycerol stimulates fat synthesis in acetate alone, but in acetate plus glucose causes no further enhancement beyond that due to glucose. Indeed, if anything, the results in all six experiments (Table 5) show that glycerol slightly inhibits the incorporation of ^{14}C into the fatty acids in presence of acetate plus glucose.

The absence of radioactivity from all the samples of glycerol isolated from the glycerides of the slices is of interest. It indicates that mammary tissue of ruminant and non-ruminant alike cannot *in vitro* incorporate acetate carbon into glycerol. This is in contrast with recent findings in the lactating animal *in vivo*. Incorporation of ^{14}C from [*carboxy*-^{14}C]-acetate into the glycerol of the milk glycerides has been observed in the lactating goat (Popják, Glascock & Folley, 1952) and rabbit (French & Popják, 1951). That this can occur in the mammary gland itself is probable since the perfused isolated bovine udder incorporates acetate carbon into lactose (Cowie *et al.* 1951). The origin of the glycerol presumably used for glyceride formation by sheep udder slices actively metabolizing acetate alone (with R.Q. > 1, as may be concluded from the results of Folley & French, 1950, and therefore probably effecting net fatty acid synthesis), is accordingly rather puzzling. It is of course possible that in these slices free fatty acids not esterified with glycerol are accumulating—and the fact that glycerol does appear to be a limiting factor in this particular system is in accord with this suggestion—but perhaps it is more likely that the necessary glycerol is formed from some precursor stored in the tissue cells.

The differences between mammary tissue from ruminants and non-ruminants in respect of their response to insulin *in vitro*, observed in our previous respiratory studies and now confirmed by isotope incorporation experiments, may be significant as regards the mechanism of the stimulating effect of insulin on fatty acid synthesis. Respiration studies indicate that mammary tissues from ruminants and non-ruminants may differ in their energy-yielding systems. Mammary tissue of the non-ruminant appears to obtain its energy from the assimilation of carbohydrate (Folley & French, 1949*a*); the ruminant udder, however, oxidizes acetate in preference to carbohydrate (Folley & French, 1950). Since fatty acid synthesis in the mammary gland is an energy-requiring process it is tempting to suggest that the insulin effect is exerted via the system which obtains energy from carbohydrate oxidation. This theory agrees with the fact that the insulin effect has been observed only with non-ruminant mammary tissue and then only in presence of glucose. It is true that Brady & Gurin (1950) found that insulin increases the incorporation of acetate carbon into the fatty acids of rat-liver slices metabolizing acetate as sole substrate, but the presence of carbohydrate stores in liver tissue from fed rats must not be overlooked in interpreting these findings. On the other hand, the similarity in the effects of glycerol and insulin on fat synthesis from acetate which emerges from the present experiments, even more clearly than from our earlier respiratory studies, suggests that, as pointed out previously (Balmain & Folley, 1951), the insulin effect in this system may possibly be mediated, partly at least, through the breakdown of glucose to glycerol which occurs in mammary tissue. Two observations on rat mammary slices are in accord with this theory: first that the effects of insulin and glycerol are not additive, and secondly that while insulin increases $-Q_{\text{glucose}}$ in acetate plus glucose, glycerol does not (Balmain & Folley, 1951). The fact that sheep udder slices in acetate plus glucose are inert not only to insulin but to glycerol as well would also fit in with this view. This very tentative hypothesis does not necessarily conflict with the findings of Chaikoff and his collaborators (Felts, Chaikoff & Osborn, 1951*a*, *b*; Osborn, Chaikoff & Felts, 1951) who showed that pretreatment of the donors with insulin diverted acetate (and the two-carbon fragment formed from pyruvate) from oxidation to fatty acid synthesis in liver slices from diabetic rats.

SUMMARY

1. The effects of insulin and of glycerol, added *in vitro*, on the incorporation of ^{14}C into the total fatty acids of slices of lactating mammary gland from the rat and sheep, incubated in [*carboxy*-^{14}C]acetate alone or with unlabelled glucose, have been studied.

2. Rat mammary gland slices incorporated no acetate carbon into the fatty acids in acetate alone, but incorporated considerable amounts in the presence of glucose. Sheep udder slices effected considerable fatty acid synthesis from acetate alone, but the utilization of acetate carbon for this purpose was greatly increased in presence of glucose.

3. Insulin did not alter the inactivity of rat mammary gland slices towards acetate as sole substrate, but in presence of glucose it considerably increased the ^{14}C found in the fatty acids. The incorporation of ^{14}C into the fatty acids of sheep udder slices was unaffected by insulin both in acetate alone and in acetate plus glucose.

4. Glycerol caused a slight incorporation of acetate carbon into the fatty acids of rat mammary gland slices in acetate alone, and in acetate plus glucose its stimulating effect on ^{14}C incorporation was as great as that of insulin. It stimulated the utilization of acetate for fatty acid synthesis by sheep udder slices in acetate alone but in acetate plus glucose its effect was slightly inhibitory.

5. Glyceride glycerol isolated as tri-*p*-nitrobenzoate from the neutral fat of the slices was not radioactive in any experiment. Mammary gland slices under our conditions do not incorporate acetate carbon into glycerol.

6. Deductions from respiratory and acid-change measurements as to the ability of mammary gland slices to utilize acetate for fatty acid synthesis *in vitro* and the effect of glucose, of insulin and of glycerol upon this process in ruminant and non-ruminant mammary tissue respectively have been confirmed. There is now little doubt that mammary gland slices are capable of effecting fatty acid synthesis from small molecules *in vitro*.

We are indebted to Dr S. K. Kon for access to the rat colony maintained by him in the Nutrition Department at this Institute; to Dr K. Hallas-Møller of Novo Terapeutisk Laboratorium, Copenhagen, for generous supplies of crystalline insulin free from glycogenolytic factor, and to Miss M. Beard for care of the sheep.

REFERENCES

Balmain, J. H. & Folley, S. J. (1951). *Biochem. J.* **49**, 663.
Balmain, J. H., Folley, S. J. & Glascock, R. F. (1951). *Nature, Lond.*, **168**, 1083.
Balmain, J. H., Folley, S. J. & Glascock, R. F. (1952). *Biochem. J.* **50**, xxix.
Balmain, J. H., French, T. H. & Folley, S. J. (1950). *Nature, Lond.*, **165**, 807.
Brady, R. O. & Gurin, S. (1950). *J. biol. Chem.* **186**, 461.
Cowie, A. T., Duncombe, W. G., Folley, S. J., French, T. H., Glascock, R. F., Massart, L., Peeters, G. J. & Popják, G. (1951). *Biochem. J.* **49**, 610.
Felts, J. M., Chaikoff, I. L. & Osborn, M. J. (1951*a*). *J. biol. chem.* **191**, 683.
Felts, J. M., Chaikoff, I. L. & Osborn, M. J. (1951*b*). *J. biol. Chem.* **193**, 557.
Folley, S. J. & French, T. H. (1949*a*). *Biochem. J.* **45**, 117.
Folley, S. J. & French, T. H. (1949*b*). *Biochem. J.* **44**, xlv.
Folley, S. J. & French, T. H. (1950). *Biochem. J.* **46**, 465.
Folley, S. J. & Watson, S. C. (1948). *Biochem. J.* **42**, 204.
French, T. H. & Popják, G. (1951). *Biochem. J.* **49**, iii.
Hills, A. G. & Stadie, W. C. (1952). *J. biol. Chem.* **194**, 25.
Osborn, M. J., Chaikoff, I. L. & Felts, J. M. (1951). *J. biol. Chem.* **193**, 549.
Popják, G. & Beeckmans, M.-L. (1950). *Biochem. J.* **46**, 547.
Popják, G., Glascock, R. F. & Folley, S. J. (1952). *Biochem. J.* **52**, 472.
Stadie, W. C. & Riggs, B. C. (1944). *J. biol. Chem.* **154**, 687.

D-Amino-acid Oxidase in the Molluscan Liver

By H. BLASCHKO and JOYCE HAWKINS
Department of Pharmacology, University of Oxford, and the Marine Biological Laboratory, Plymouth

(*Received* 23 *January* 1952)

The enzyme D-amino-acid oxidase was first found in mammalian liver and kidney (Krebs, 1933); it occurs in all vertebrates that have been examined. A review on D-amino-acid oxidases by Krebs (1948) refers to the occurrence of an enzyme of this type in bacteria and in certain strains of the mould *Neurospora*.

We have recently briefly reported that D-amino-acid oxidase is present in the liver of *Octopus vulgaris* and of *Sepia officinalis* (Blaschko & Hawkins, 1951). This appears to be the first time that the enzyme has been found in invertebrates. We now wish to report some observations on the occurrence of D-amino-acid oxidase in other classes of molluscs and also to describe some of the properties of this enzyme.

MATERIALS AND METHODS

Animals. Specimens of *Sepia officinalis* and *Octopus vulgaris* were dissected in Plymouth. The livers were immediately frozen in solid CO_2 and taken to Oxford in a thermos flask. They were then stored at $-10°$.

In the other species examined (*Mytilus, Anodonta, Helix*) the liver is not as well defined a structure as in the two species of cephalopods, and tissue used included parts of the alimentary canal. *Mytilus edulis* was also obtained from

Plymouth and dissected in Oxford. A few specimens of *Anodonta* were collected locally, and specimens of a species of *Helix* (possibly *aspersa*) were collected in a garden in Oxford.

The enzyme preparation. Homogenates were prepared in 0·067 M-sodium phosphate buffer of pH 7·4; usually 1 g. of tissue was homogenized in a total volume of 5 ml. The preparation of an acetone-dried powder of *Octopus* liver will be described later.

Measurement of enzymic activity. This was determined manometrically. The main compartment of a conical flask contained 1·6 ml. of homogenate, the side bulb 0·4 ml. of either water or of a solution of the amino-acid to be tested. The concentration of the amino-acid solution was 0·05 M, when the D-amino-acid was used, and 0·1 M when the DL-amino-acid was used. The inner tube contained a filter paper and 0·3 ml. of N-KOH. The gas phase was O_2, and the temperature, unless otherwise stated, was 37·5°. Readings were usually taken at 5 min. intervals for 30 min.; the enzymic activity was usually calculated from the O_2 uptake during the first 15 min. period of incubation.

Some of the amino-acids did not dissolve completely; this was most marked with tyrosine, cystine and 2-amino-*n*-octanoic acid, which were added as suspensions. Solutions of the dicarboxylic acids, and of the dihydrochlorides of the basic amino-acids, were neutralized before use.

RESULTS

Cephalopods

The experiments on D-amino-acid oxidase of *Sepia* liver were carried out early in 1951, after the livers had been stored for about 4 months. It is therefore possible that the enzymic activity of the fresh organs had been higher. In the first experiment valine was used as substrate, and the oxygen uptakes (μl. O_2) recorded in 30 min. were: without added amino-acid, 38; additional uptake with D-valine, 28; additional uptake with L-valine, 3. In view of the large blank, the oxidation of L-valine cannot be considered significant.

D-Leucine was also oxidized by the *Sepia* enzyme, and in all the later experiments, one flask was included in which the oxidation of D-leucine was measured; this compound serves as basis of comparison. Table 1 contains results with all those amino-acids tested which can be considered as substrates of oxidation in *Sepia*; the figures in the last column give the relative rates of oxidation compared with that of D-leucine. A number of other amino-acids were tested, but these did not significantly raise the oxygen uptake above that found in the blank. They were: D-alanine, DL-α-amino-*n*-butyric acid, DL-serine, DL-threonine, DL-proline, DL-aspartic acid, DL-ornithine, DL-lysine, D-histidine, DL-cystine and DL-homocystine. It must be pointed out, however, that in view of the large oxygen consumption of the 'enzyme blank', it is quite possible that some of these compounds are oxidized, but at a slower rate.

The experiments on *Octopus* enzyme described here were carried out in the summer of 1951, on a liver which had been dissected only a few days earlier. The liver weighed about 35 g. Part of the organ was used in the preparation of several homogenates; the remainder, 12·8 g., was converted into an acetone-dried powder.

Altogether eight experiments were carried out, using different homogenates. The enzyme blank in the first 15 min. varied between 22 and 29 μl. O_2, the additional oxygen uptake with D-leucine, which again served as a standard of comparison, between 30 and 45·5 μl. These experiments are shown in Table 2.

It can be seen from Table 2 that all the unbranched monoamino-monocarboxylic acids were oxidized; the maximum rate of oxidation in this series occurred with α-amino-*n*-butyric acid. Of the branched acids, valine, leucine and isoleucine were oxidized, but α-amino*iso*butyric acid was not oxidized. It is interesting that histidine (the DL form) was oxidized by the *Octopus* preparation. The result with DL-lysine must be considered as doubtful. DL-Ornithine was not oxidized in one experiment, DL-threonine was not oxidized in two experiments.

Table 1. *Oxidation of* D-*amino-acids in homogenates of* Sepia *liver*

Exp. no.	1	2	3	4	5	6	7	Mean, as % of oxidation of D-leucine
Amino-acid	Additional O_2 uptake in 15 min. (μl.)							
DL-Norvaline	—	15·5	—	—	—	—	—	143
DL-Norleucine	—	11·5	7·5	—	—	—	—	110
D-Valine	—	4·5	3·5	—	—	—	—	48
D-Leucine	41·5	11	6·5	10	12·5	8	8	100
D-Isoleucine	28	6·5	4·5	—	—	—	—	65
DL-Glutamic acid	—	—	—	12·5	15	10·5	9	120
D-Tryptophan	—	16	7	—	—	—	—	126
D-Phenylalanine	—	13	7·5	—	—	—	—	117
DL-Methionine	—	18	14	—	—	—	—	189

In one additional experiment in which D-leucine was not used, the 15 min. figures for oxygen uptake (μl.) were: without added substrate, 29; additional uptake with D-phenylalanine, 75; additional uptake with DL-phenylalanine, 58·5; additional uptake with L-phenylalanine, 7.

Part of the same *Octopus* liver was converted into an acetone-dried powder; an extract was prepared from the powder following the procedure of Bender & Krebs (1950), except that the extract was not obtained by filtration but by centrifugation for 15 min. at 0°. This gave an almost clear supernatant fluid. We used the sodium pyrophosphate buffer of pH 8·4 described by Warburg & Christian (1938) for the extraction.

A sample (1 ml.) of this extract and 0·6 ml. of the pyrophosphate buffer were used in each flask. The genates were prepared by adding 2 ml. of cold 0·67 M-sodium phosphate buffer of pH 7·4 to each g. of tissue and grinding thoroughly. In the manometric experiment, each flask contained 0·5 ml. of the homogenate plus 1·1 ml. of the buffer. The temperature in these experiments was 17°; the gas phase was O_2.

Altogether five experiments were carried out with preparations from *Mytilus*. No significant oxygen uptake was noted with valine, leucine, proline, threonine, glutamic acid and tryptophan.

Table 2. *Oxidation of* D-*amino-acids in* Octopus *liver*

Exp. no.	1	2	3	4	5	6	7	8	Mean, as % of oxidation of D-leucine
Amino-acid	Additional O_2 uptake in 15 min. (μl.)								
DL-Alanine	—	44·5	—	—	—	—	—	35	100
DL-α-Amino-*n*-butyric acid	—	—	—	66·5	—	—	—	70·5	188
DL-Norvaline	49·5	—	—	—	—	—	—	56	165
DL-Norleucine	30	—	—	—	—	52	—	60	132
DL-2-Amino-*n*-octanoic acid	—	—	—	—	19	—	—	21	56
DL-Valine	—	—	—	32·5	—	—	16	—	64
D-Leucine	30	45·5	34·5	39	38	38·5	35·5	34	100
D-Isoleucine	—	—	—	25·5	—	—	26·5	—	70
DL-Aspartic acid	—	—	14	—	19·5	—	—	—	46
DL-Glutamic acid	38	—	—	—	—	—	31·5	—	108
DL-Serine	—	21	—	—	—	16·5	—	—	44
DL-Proline	—	—	30	—	—	—	—	29·5	87
D-Tryptophan	4	—	—	17·5	12·5	—	—	—	30
DL-Histidine	—	—	37	—	—	—	—	42·5	116
DL-Lysine	4	—	—	—	—	0	—	—	—
DL-Methionine	39	—	—	—	—	—	45·5	—	129

Table 3. *Oxidation of* D-*amino-acids in homogenates of* Helix *liver*

Exp. no.	1	2	3	4
Amino-acid	Additional O_2 uptake in 15 min. (μl.)			
DL-Alanine	—	—	22	11
DL-α-Amino-*n*-butyric acid	—	—	35·5	33·5
DL-Norvaline	—	—	44·5	47
DL-Norleucine	—	—	9	11·5
DL-2-Amino-*n*-octanoic acid	—	—	5·5	0
D-Leucine	78	72	59	44·5
D-Isoleucine	—	—	—	3
D-Phenylalanine	49·5	38·5	—	—
DL-Methionine	61·5	50	—	—

amounts of oxygen (μl.) consumed in 30 min. were: without added substrate, 7; additional uptake with DL-glutamic acid, 77; L-glutamic acid, 7·5; DL-tyrosine, 14·5; L-tyrosine, 6.

The experiment shows that the rapid oxygen uptake with glutamic acid occurred only when the D form was present. This makes it likely that the oxidation was catalysed by D-amino-acid oxidase and not by another enzyme. The oxygen uptake in the flask with DL-tyrosine was larger than in that with L-tyrosine; the slight oxidation of L-tyrosine may possibly have been due to tyrosinase which is known to occur in *Octopus*.

Lamellibranchs

Mytilus. The 'livers' from a number of specimens were dissected, weighed and frozen in a mortar at −10°. Homo-

There was an oxygen uptake in two experiments with DL-methionine (73·5 and 74·5 μl. O_2 respectively in 15 min.), and in one experiment with DL-serine (17·5 μl. O_2 in 15 min.). With the unbranched monoamino acids the oxygen consumed in 15 min. was:

	Exp. 1 (μl. O_2)	Exp. 2 (μl. O_2)
Blank	4	4
DL-Alanine	6·5	0
DL-α-Amino-*n*-butyric acid	13·5	27
DL-Norvaline	33	44
DL-Norleucine	47	43
DL-2-Amino-*n*-octanoic acid	0	11·5

Anodonta. Two experiments were carried out with the 'liver' homogenate. In the first experiment there was no oxygen uptake with D-leucine,

D-phenylalanine and DL-methionine; in the second experiment with D-leucine, DL-phenylalanine, DL-norvaline and DL-methionine there was again no oxygen uptake. The blank oxygen uptakes in 15 min. at 22° were 10·5 and 15·5 μl. respectively.

Gastropod (Helix)

The results of four experiments with the 'liver' homogenate are set out in Table 3. The blank oxygen uptake in these experiments varied from 4·5 to 14·5 μl., and many amino-acids were found to be oxidized. The result with 2-amino-*n*-octanoic acid and with isoleucine must be considered doubtful. In Exp. 4, DL-cystine, DL-homocysteine and DL-ornithine were also tested; they were not oxidized.

DISCUSSION

The experiments here reported show that in four of the five species of molluscs examined, the liver contains a D-amino-acid oxidase akin to that found in vertebrates. The enzyme occurred in all the classes examined: cephalopods, lamellibranchs and gastropods. It is of interest to compare the properties of the preparation studied in this paper with those of the mammalian enzyme. Bender & Krebs (1950) have recently examined the substrate specificity of the sheep-kidney enzyme. In many ways the molluscan enzymes resemble the sheep-kidney enzyme, but there are some differences not only between the sheep and the molluscs, but also differences from one mollusc to another. Points of similarity are that many monoamino-monocarboxylic acids are readily attacked by the sheep enzyme and by the molluscan enzyme; the basic amino-acids are not so readily oxidized. Serine is oxidized by the sheep and by *Octopus* and so is proline. Both methionine and phenylalanine are readily attacked in mammals and in molluscs. Of the differences, one may be noted: the *Octopus* enzyme oxidizes D-glutamic acid, which is not a substrate of the sheep enzyme.

There are differences between the different molluscan preparations in the relative rates of oxidation in the homologous series

$$CH_3(CH_2)_nCHNH_2COOH.$$

Of this series, alanine ($n=0$), α-amino-*n*-butyric acid ($n=1$), norvaline ($n=2$), norleucine ($n=3$) and 2-amino-*n*-octanoic acid ($n=5$) were available. In *Octopus* all members of the series were oxidized; the maximum rate of oxidation was found with α-amino-*n*-butyric acid ($n=1$); in *Sepia*, norvaline ($n=2$) and norleucine ($n=3$) were the only members that were oxidized at a measurable rate; in *Helix*, where all members were oxidized, the maximum rate was with norvaline ($n=2$). In *Mytilus*, norvaline and norleucine were oxidized most readily, and with both alanine ($n=0$) and 2-amino-*n*-octanoic acid ($n=5$) there was little or no oxygen uptake. These results show that at first there is a rise of the oxidation rate with increasing chain length, but that with a further increase in chain length the rate falls off. This might be explained by the lower solubility of the higher members of the series, but it is interesting that in each species the specificity pattern is different and the maximum rate of oxidation occurs with a different member of the series.

The structure and function of the liver in molluscs differs fundamentally from that in vertebrates. In fact, in different molluscan species the liver has an entirely different character. This subject is reviewed by Yonge (1937). In *Octopus* and *Sepia* the liver is a parenchymatous organ which is separated from the gut, as it is in vertebrates. In the other three species examined (*Mytilus*, *Anodonta* and *Helix*), the 'liver' is not separated from the gut, and each part of the tissue contains not only secretory elements, but also parts of the intestinal lumen. An interesting outcome of our observations is that the presence or absence of the enzyme does not coincide with these differences in structure and function, but that not only the cephalopods, but also *Mytilus* and *Helix*, contain the enzyme. The possibility that in the two latter species intestinal bacteria contribute to the enzymic activity cannot be excluded, but it is of interest that the enzyme in these two species is akin to the oxidase in vertebrates and in cephalopods where the danger of bacterial contamination seems remote.

The enzyme was not found in *Anodonta*. This may have been due to unsuitable experimental conditions, but these did not differ from those in which the presence of the enzyme in other species was easily demonstrated. It is of interest that in marine lamellibranchs (*Ostrea* and *Mytilus*) the concentration of non-protein amino-acids in the muscle tissue is high, but that in the fresh-water species, *Anodonta*, the amino-acid content is very low (Duchâteau, Sarlet, Camien & Florkin, 1952). This seems to make it worth considering whether the oxidase is in some way connected with the metabolism of the amino-acids in the tissues.

It would clearly be of interest to extend this survey of D-amino-acid oxidase to other phyla, as a better knowledge of its distribution may help towards an understanding of its function in the animal body, which is still unknown (see Krebs, 1948). It has been suggested (Warburg, 1948) that *in vivo* the enzyme is concerned in the synthesis of L-amino-acids rather than in the breakdown of the D forms. However, D-amino-acids are now known to occur in nature more commonly than had been believed and this makes it less surprising that an

enzyme dealing with these substances is so widespread. Possibly there are animals in which D-amino-acids are metabolized either as constituents of the diet or as products of intermediate metabolism.

SUMMARY

1. The enzyme D-amino-acid oxidase occurs in the liver of two cephalopods, *Sepia officinalis* and *Octopus vulgaris*. The substrate specificity of the enzyme has been studied in tissue homogenates. The *Octopus* enzyme is also present in the extract of an acetone-dried powder of the liver.

2. The enzyme was also found in the 'liver' of a gastropod species, *Helix*.

3. The 'livers' of two lamellibranchs were examined. The enzyme was found in *Mytilus edulis*, but it was not found in *Anodonta*.

The authors are grateful to the Director and Staff of the Marine Biological Laboratory for help and facilities offered. They also wish to thank Miss Jean Himms for technical assistance.

REFERENCES

Bender, E. A. & Krebs, H. A. (1950). *Biochem. J.* **46**, 210.
Blaschko, H. & Hawkins, J. (1951). *Biochem. J.* **49**, xliv.
Duchâteau, G., Sarlet, H., Camien, M. N. & Florkin, M. (1952). *Arch. int. Physiol.* (in the Press).
Krebs, H. A. (1933). *Hoppe-Seyl. Z.* **219**, 191.
Krebs, H. A. (1948). *Biochem. Soc. Symp.* **1**, 2.
Warburg, O. (1948). *Wasserstoffuebertragende Fermente.* Berlin: W. Saenger.
Warburg, O. & Christian, W. (1938). *Biochem. Z.* **298**, 150.
Yonge, C. M. (1937). *Biol. Rev.* **12**, 87.

The Effect of Age on the Bulk Protein Composition of *Chlorella vulgaris*

By L. FOWDEN
Department of Botany, University College, Gower Street, London, W.C. 1

(*Received* 30 *January* 1952)

The effect of time of harvesting upon the proportions of the total protein nitrogen found as amide N, 'basic N' and monoamino N in the leaves of higher plants has been studied by Chibnall (1922, 1926) and Pearsall (1931). Such investigations have been continued by Lugg & Weller (1941, 1948) who determined the percentages of certain amino-acids (tryptophan, methionine and cystine-cysteine) in leaf-protein fractions. Methionine and cystine contents of the proteins of pasture plants harvested at different times have also been determined by Smith & Wang (1941).

The variation of protein composition with age in members of the lower plant orders has been little investigated. Several workers (Camien, Salle & Dunn, 1945; Freeland & Gale, 1947) have studied the protein composition of various micro-organisms, and the effects produced by variations in the culture media. The effect of the age of the culture upon the protein composition was not, however, investigated, the cultures normally being harvested during their exponential phase of growth. Fowden (1951*a*) has reported the composition of the bulk protein of the alga *Chlorella vulgaris* during its phase of exponential growth; Mazur & Clark (1938, 1942) earlier reported the protein composition of several algae at undefined stages of their growth.

The present paper presents the results of an investigation of the protein composition of *Chlorella* and includes complete analyses of protein fractions obtained from cultures of widely differing ages. In addition to contributing to knowledge of the nitrogen metabolism of this alga, the analyses can be expected to provide data for assessing the merits of *Chlorella* cultures of different ages if used as a source of food, as envisaged by several authors (Spoehr & Milner, 1948; Meier, 1949; Pearsall & Fogg, 1951; Geoghegen, 1951).

EXPERIMENTAL

Growth of alga. C. vulgaris was grown on the medium of Pearsall & Loose (1936) to which was added 1 ml./l. of trace-element solution A4 (Arnon, 1938) containing B, Mn, Cu and Zn. Into each one of a batch of penicillin-culture flasks were introduced 500 ml. of the medium; they were then autoclaved for 20 min. at a pressure of 12 lb./sq. in. The medium, which was heavily buffered with phosphate, usually had a pH of 6·1–6·2 at this stage. Each flask was then inoculated with 1 ml. of an actively growing *Chlorella* culture containing about 5000 cells/cu.mm. The flasks, now containing about 10 cells/cu.mm., were maintained at 20±2° under a continuous uniform illumination of 150 ft.c. Growth was allowed to proceed for various periods of time, the flasks being gently shaken twice per day.

Harvesting. At selected intervals of time, the contents of one flask were harvested. The flask was gently shaken to obtain a uniform suspension and a few ml. of the culture were removed for haemocytometer cell counts. The remainder of the culture was centrifuged at 500 *g* for 5 min., after which the supernatant medium could be decanted without disturbing the packed cells. The supernatants obtained in this way were preserved under toluene at 3° until the necessary analyses could be performed. The cells were washed twice by centrifugation with distilled water. A small sample was then removed, dried at 95°, ground to a fine powder, and then stored for determination of total N. The remaining cells were immediately treated as described later for protein extraction. During the first few days of growth, the contents of several flasks had to be combined in order to obtain sufficient material for a successful protein extraction to be performed. The maximum age of any culture examined was 5 weeks. Additional culture flasks were harvested at intervals to determine more accurately the rate of growth of the *Chlorella* and the rate of absorption of N from the medium by the cells. No attempt was made to extract the protein from the cells in these additional cultures.

Nitrogen absorption from the medium. Equal amounts of NH_3-N and NO_3^--N were supplied as NH_4NO_3 in the medium, this being the only source of N. The assimilation of each form of N by the *Chlorella* was determined as the difference between the amounts originally present in the media and those remaining after harvesting the cells.

The NH_3-N present in 5 ml. of each medium was removed by distillation with MgO in a Markham still, the NH_3 being collected in the boric acid-indicator mixture of Conway (1947), and determined by titration against 0·01 N-HCl.

The NO_3^--N present in 0·25 ml. of each medium was determined colorimetrically using phenoldisulphonic acid (Snell & Snell, 1949). After making alkaline, the volumes were made up to 25 ml., and the absorption measured at $\lambda = 440$ mμ. in a Unicam spectrophotometer. It was observed that the optical density of the colour produced in the presence of the other constituents of the medium was less than that produced from the same amount of NO_3^--N when NH_4NO_3 was used in pure solution. Thus the optical densities corresponding to known amounts of nitrate had to be determined by difference between the intensities of the colours produced from 0·25 ml. of each medium, and those obtained after the addition of standard amounts of NO_3^--N to further 0·25 ml. samples of the media.

Protein extraction, hydrolysis and analysis. The procedures employed for the extraction, hydrolysis and subsequent chromatographic amino-acid analysis of the protein fractions from the harvested cells were those described in an earlier paper (Fowden, 1951*a*) with the following slight modifications and additions. In the case of cultures having a cell count greater than 35 000/cu.mm., the freshly harvested cells were suspended in a volume of borax solution somewhat greater than the 35 ml. used previously, in order to maintain the high percentage of cell breakdown during the shearing process. Total N determinations were performed on each of the protein fractions isolated.

All the protein fractions were hydrolysed without any trace of humin formation, with the exception of one fraction which had a very low N content of 8·6%. In this case a small amount of humin was precipitated during the course of the hydrolysis, but this resulted in a loss of less than 1% of the total protein nitrogen.

In order to confirm the histidine contents of the different protein fractions obtained using the paper-chromatographic method of assay, additional estimates were obtained using the modified Pauly colorimetric method of Macpherson (1946). The method was subject to interference from tyrosine, which produced a colour similar to that given by histidine with the Pauly reagent. The sensitivity for tyrosine was found to be only 28% of that obtained with histidine, and so accurate corrections could be applied to the histidine values for the known amounts of tyrosine in each protein hydrolysate.

Proline estimations were carried out after the chromatographic separations using the colorimetric method of Fowden (1951*b*), the final colours being read at $\lambda = 440$ mμ. Although the sensitivity of this method for proline was only one-third of that obtained for the α-amino-acids, the method was found to be more reliable than the copper colorimetric method used previously for proline assay (Fowden, 1951*a*).

Total N determinations. These were done by a micro-Kjeldahl method.

Amide N. Values were obtained by estimating the NH_3 produced during hydrolysis with N-H_2SO_4 for 3 hr. at 100° by the nesslerization procedure of Umbreit, Burris & Stauffer (1945).

RESULTS

Growth of Chlorella. No significant lag phase in the growth of the *Chlorella* was observed after the initial inoculation of the culture flasks. The cell count increased in an exponential manner during the first 7 days of growth, when the cell concentration was about 15 000/cu.mm. After this time, the frequency of cell division decreased, maximum growth being recorded at about 20 days when the cell count had reached approximately 68 000/cu.mm. In older cultures, cell breakdown had commenced with a consequent reduction in the cell counts, which at the end of the 5-week growth period had fallen to 40 000/cu.mm.

The nitrogen available in the medium appeared to determine, at least in part, the amount of growth possible; virtually all the nitrogen present in the medium had been assimilated by the cells in the first 13 days of growth, when the cell count had reached 58 000/cu.mm. The further growth beyond this point must have occurred by a process in which some of the limited store of soluble nitrogenous compounds within the cells was utilized for new protein synthesis.

During the early stages of growth, the NH_3-N was used much more rapidly than the NO_3^--N. At the end of the phase of exponential growth (7 days) one-half of the NH_3-N supplied had been absorbed, whilst only about one-eighth of the total NO_3^--N had left the medium. The remaining NH_3-N was absorbed by the end of the 10th day of growth. The rate of NO_3^--N utilization then increased markedly until nitrate also was completely removed from the medium. The preferential absorption by the cells of NH_3-N resulted in a decrease in the pH of the medium of about 0·5 unit, the fall being following by

a slight rise of pH as the nitrate concentration decreased.

Table 1 presents the data obtained during the growth of the *Chlorella* showing the numbers of cells and the absorption of NH_3-N and NO_3^--N from the medium.

Total nitrogen determinations on whole dried cells indicated that protein formed 30 % of the cell dry weight during the phase of exponential growth. During the later stages of growth the total cell nitrogen content decreased to about two-thirds of its former value. Soluble cell nitrogen extracted with 70 % (v/v) aqueous ethanol, when expressed as a percentage of the total cell nitrogen, remained fairly constant during the whole period of growth studied.

Table 1. *The growth rate of* Chlorella vulgaris, *the levels of combined nitrogen remaining in the medium, and the changes in* pH *of medium*

Time of growth (days)	Cell count (per cu.mm.)	NH_3-N remaining in medium (μg. N/ml.)	NO_3^--N remaining in medium (μg. N/ml.)	pH of medium
0	12	131	131	6·20
3	525	129	129	6·09
4	1 250	—	—	—
5	2 700	116	127	6·05
6·7	14 560	66	117	5·86
8·8	34 000	5	91	—
9·9	40 400	0	80	5·66
12·9	58 000	0	8	5·90
16·7	66 000	0	0	5·89
21	68 000	0	0	5·86
28	52 000	0	0	—
35	40 000	0	0	—

Extraction of protein fractions. Protein was extracted from freshly harvested *Chlorella* cells at six different stages during their growth. The protein fractions isolated after the cells had been broken by shearing contained 80–85 % of the total nitrogen released from the cells which had been broken. Since 10–15 % of the total nitrogen of the cells was present as soluble nitrogenous constituents, the fractions isolated were considered to be representative of the whole cell proteins. The nitrogen contents of the protein fractions isolated were generally slightly greater than 10 % of their dry weight.

Table 2. *The total and soluble nitrogen contents of* Chlorella vulgaris, *and nitrogen contents of protein fractions isolated*

Age of culture (days)	Total N of dried cells (g. N/100 g.)	Soluble N of dried cells (g./100 g. total N)	N in protein fractions isolated (g. N/100 g.)
5	5·8	10·1	10·3
8·8	3·9	—	8·6
12·9	3·6	10·3	10·5
21	3·8	—	10·0
28	3·6	13·1	10·5
35	3·7	—	10·1

Table 2 contains the data obtained from nitrogen analyses on whole dried cells and on the protein fractions isolated.

Composition of bulk protein fractions. Four of the protein fractions were subjected to amino-acid analysis. These fractions had been isolated from *Chlorella* cells representative of the four main stages of growth observed, namely from cells harvested during the exponential phase of growth (culture *A*), at the end of this phase (culture *B*), at the time of maximum growth (culture *C*), and during the senescent breakdown stage (culture *D*). In cultures *A* and *B* the cells had used ammonia as their main nitrogen source, whilst both the ammonia and the nitrate had been completely absorbed at the stage of maximum growth.

Table 3 presents the results of the amino-acid analyses performed on the hydrolysates of the protein fractions. With the exception of the values given for cystine and tryptophan, all estimates were obtained by the method of quantitative amino-acid paper chromatography. Cystine and tryptophan determinations were made, using specific colorimetric methods. Histidine values obtained by the chromatographic and modified Pauly methods are given separately in the Table. The figures presented are the mean values of four determinations in the case of amino-acids estimated by the chromatographic method, whilst cystine, tryptophan, histidine (by modified Pauly) and amide N values are the means of duplicate determinations. The standard deviations from the mean values are given in parentheses.

DISCUSSION

In form, the growth curve of the *Chlorella*, which reached a maximum value at about the 20th day, resembled that obtained by Pearsall & Loose (1936) for the same species. The maximum cell concentration observed was, however, over twice that found by the earlier workers. Temperature, light intensity

and the amounts of available nitrogen and other inorganic salts are probably the factors controlling the rate and total amount of growth under the conditions used. In the present investigation, all the nitrate supplied in the medium was absorbed by the *Chlorella* cells during their growth, whereas Pearsall & Loose (1936) found that only one-third of the nitrate had been absorbed from the medium by the time growth ceased. This difference in the utilization of nitrate by the cells was probably the most important factor in determining the differences in the cell concentrations at the time of maximum growth.

determinations. The histidine contents, however, increased steadily to a value in culture *D* more than 1·5 times that in culture *A*; smaller increases in the percentages of alanine and lysine similarly occurred. In addition, ageing of the cultures was marked by a decrease of one-fifth in the arginine values, and a slight fall in the amide N figures.

The fact that the amino-acid compositions of the bulk proteins of *Chlorella* remained virtually unchanged with increasing age cannot be regarded as proof that the relative amounts of the individual proteins present within the cells was unaltered.

Table 3. *The amino-acid compositions of bulk protein fractions isolated from cultures of* Chlorella vulgaris *during different phases of growth*

(Figures in brackets are standard deviations.)

Culture	*A*	*B*	*C*	*D*
Age of culture (days)	5	8·8	21	35
Cell count (per cu.mm.)	2700	34 800	68 000	40 000
	g. amino-acid N/100 g. protein N			
Aspartic acid	6·0 (0·3)	5·7 (0·4)	6·5 (0·1)	6·0 (0·3)
Glutamic acid	7·9 (0·5)	6·9 (0·3)	7·5 (0·2)	7·8 (0·4)
Serine	3·4 (0·2)	3·0 (0·2)	2·9 (0·2)	3·6 (0·1)
Threonine	3·0 (0·2)	3·5 (0·2)	3·5 (0·2)	2·7 (0·2)
Glycine	6·3 (0·1)	5·9 (0·4)	5·8 (0·3)	5·7 (0·3)
Alanine	7·9 (0·2)	8·8 (0·3)	9·0 (0·4)	9·2 (0·3)
Valine	5·6 (0·3)	5·2 (0·3)	5·4 (0·3)	5·9 (0·1)
Leucine	6·1 (0·1)	5·6 (0·4)	5·6 (0·4)	6·0 (0·2)
Isoleucine	3·1 (0·1)	3·4 (0·2)	3·2 (0·2)	3·5 (0·2)
Phenylalanine	2·3 (0·1)	2·3 (0·1)	2·3 (0·1)	2·2 (0·1)
Tyrosine	2·6 (0·1)	2·6 (0·1)	2·8 (0·1)	2·7 (0·1)
Proline	4·6 (0·2)	4·6 (0·3)	4·5 (0·2)	4·7 (0·2)
Tryptophan	2·1	2·1	2·0	1·8
Methionine	1·4 (0·1)	1·4 (0·1)	1·3 (0·1)	1·2 (0·1)
Cystine	0·15	0·2	0·2	0·2
Arginine	17·8 (1·0)	16·0 (0·7)	16·6 (1·1)	14·0 (1·0)
Histidine	3·6 (0·1)	4·1 (0·3)	4·9 (0·2)	6·0 (0·3)*
	3·5	3·6	4·8	5·2 †
Lysine	8·3 (0·4)	8·3 (0·3)	8·9 (0·4)	9·1 (0·3)
Amide N	6·4	6·8	6·1	5·8
Total N	98·6	96·2	99·0	97·7

* Chromatographic values. † Modified Pauly values.

According to current theories, an absence of manganese from plant tissues results in a number of abnormalities, one of which is an impaired efficiency of nitrate reduction. It is therefore possible that in the earlier experiments, where no trace elements were added to the culture medium, the incomplete utilization of nitrate may have been due to a deficiency of manganese within the cells.

The analyses presented in Table 3 show good agreement with those published earlier (Fowden, 1951*a*) for the protein extracted from *Chlorella* cells during the exponential growth phase, although the proline value then reported is now considered to have been overestimated. Table 3 also shows that there was little change in the composition of the bulk protein with the age of the various cultures, the percentages of most amino-acids remaining constant within the limits of the probable errors of the

Changes may have occurred in the concentrations of proteins of similar amino-acid composition, whilst the possibility of a more general change in the protein balance within the cells cannot be completely overlooked. The increasing histidine contents may then be interpreted as arising from a synthesis of one or more proteins relatively rich in this amino-acid during the later stages of growth.

The investigations have so far studied only the amino-acids bound in the form of protein, which accounts for about 85% of the total cell nitrogen. Whether the age of the culture has any more marked effect on the concentrations of the soluble nitrogenous compounds, particularly the free amino-acids and peptides within the cells, awaits further investigation.

Several papers have already been mentioned in

which *Chlorella* has been envisaged as a possible source of dietary protein. When the results in Table 3 are examined from this angle, it is evident that the quality of the protein is nearly independent of culture age. Thus harvesting of the cells could be arranged at a time which from an industrial viewpoint would be most economical. The ten 'essential' amino-acids of Rose account for 53% of the total protein nitrogen, or 50% of the total weight of all amino-acids released during protein hydrolysis. Therefore, although the content of cystine is low, the remaining essential amino-acids are present in *Chlorella* protein in proportions comparing very favourably with those for other plant proteins.

SUMMARY

1. The unicellular alga *Chlorella vulgaris* was grown in pure culture. The utilization by the cells of NH_3-N and NO_3^--N supplied in the medium was examined, growth being observed to continue until the supplies of nitrogen became exhausted.

2. Bulk protein samples representative of the whole cell proteins were isolated from cultures of different ages, and their amino-acid compositions determined by a paper-chromatographic technique. It was found that culture age had little effect on the protein composition except for a significant increase in the histidine contents with increasing age of the cells, and slight changes in alanine, lysine, arginine and amide N contents.

3. The 'essential' amino-acids accounted for 50% by weight of the total amino-acids released during hydrolysis of the proteins, although the cystine contents were uniformly low.

My thanks are due to Mr P. J. Syrett for providing actively growing cultures of *Chlorella vulgaris*, and to Prof. W. H. Pearsall, F.R.S., for his interest in this work.

REFERENCES

Arnon, D. I. (1938). *Amer. J. Bot.* **25**, 322.
Camien, M. N., Salle, A. J. & Dunn, M. S. (1945). *Arch. Biochem.* **8**, 67.
Chibnall, A. C. (1922). *Biochem. J.* **16**, 344.
Chibnall, A. C. (1926). *Biochem. J.* **20**, 108.
Conway, E. J. (1947). *Microdiffusion Analysis and Volumetric Error*, p. 86. London: Crosby Lockwood.
Fowden, L. (1951*a*). *Biochem. J.* **50**, 355.
Fowden, L. (1951*b*). *Biochem. J.* **48**, 327.
Freeland, J. C. & Gale, E. F. (1947). *Biochem. J.* **41**, 135.
Geoghegen, M. J. (1951). *Nature, Lond.*, **168**, 426.
Lugg, J. W. H. & Weller, R. A. (1941). *Biochem. J.* **35**, 1099.
Lugg, J. W. H. & Weller, R. A. (1948). *Biochem. J.* **42**, 412.
Macpherson, H. T. (1946). *Biochem. J.* **40**, 470.
Mazur, A. & Clark, H. T. (1938). *J. biol. Chem.* **123**, 729.
Mazur, A. & Clark, H. T. (1942). *J. biol. Chem.* **143**, 39.
Meier, R. L. (1949). *Chem. Engng News*, **27**, 3112.
Pearsall, W. H. (1931). *J. exp. Biol.* **8**, 279.
Pearsall, W. H. & Fogg, G. E. (1951). *Department of Scientific and Industrial Research Food Science Abstracts*, **23**, 1.
Pearsall, W. H. & Loose, L. (1936). *Proc. roy. Soc.* B, **121**, 451.
Smith, A. M. & Wang, T. (1941). *Biochem. J.* **35**, 404.
Snell, F. D. & Snell, C. T. (1949). *Colorimetric Methods of Analysis*, 3rd ed., vol. 2, p. 792. New York: Van Nostrand.
Spoehr, H. A. & Milner, H. W. (1948). *Rep. Dir. Div. Plant Biol., Carneg. Instn Wash. Yearb.* **47**, 100.
Umbreit, W. W., Burris, R. H. & Stauffer, J. F. (1945). *Manometric Methods*, p. 103. Minneapolis: Burgess.

The Biosynthesis of L-Cystine and L-Methionine Labelled with Radioactive Sulphur (^{35}S)

By R. B. WILLIAMS and R. M. C. DAWSON
Neuropsychiatric Research Centre, Whitchurch Hospital, Cardiff

(*Received* 20 *February* 1952)

The sulphur-containing amino-acids labelled with radioactive sulphur (^{35}S) are usually administered in biological experiments as a racemic mixture of their optical isomers. As the metabolism of L-amino-acids in animals generally differs from that of the D forms (Neuberger, 1948; Graham *et al.* 1950; Camien, Malin & Dunn, 1951), it may be desirable in certain experiments to use the labelled amino-acids as their naturally occurring isomers. The chemical synthesis of cystine or methionine from labelled sulphate ions followed by the resolution of the isomers is a somewhat tedious procedure and a biological synthesis may be preferred.

During the growth of baker's yeast, inorganic sulphate is easily assimilated and is a better sulphur source than organically combined sulphur (Sugata & Koch, 1935; Schultz & McManus, 1950). It seemed likely therefore that the proteins of yeast grown in a medium containing labelled sulphate ions would serve as a convenient source for the isolation of labelled L-cystine and L-methionine. This paper gives details of the preparation of these

labelled amino-acids from the proteins of yeast grown in a radioactive medium. Glutathione labelled with radioactive sulphur has also been prepared from a similar yeast preparation, but as the method followed closely one published during this investigation (Woodward, 1951) no description will be given.

METHODS AND RESULTS

Growth of yeast and preparation of crude yeast protein

Baker's yeast (*Saccharomyces cerevisiae*) was grown in a synthetic medium similar to that used by Olsen & Johnson (1949). 1 l. of the medium contained the following: glucose, 20 g.; $(NH_4)_2HPO_4$, 3·5 g.; KH_2PO_4, 0·2 g.; $MgSO_4.7H_2O$, 0·25 g.; sodium citrate, 1·0 g.; L-asparagine monohydrate, 2·5 g.; biotin, 10 μg.; calcium pantothenate, 0·5 mg.; inositol, 10 mg.; thiamine, 6 mg.; pyridoxin, 1 mg.; Zn^{++} (acetate), 400 μg.; Fe^{+++} (chloride), 150 μg.; Cu^{++} (chloride), 25 μg.

The medium was adjusted to pH 5 with H_3PO_4 and warmed to 30°. Radioactive sulphur (1 mc./l. as carrier-free SO_4^{2-}) was then added. Sterilization of the medium was found to be unnecessary if it was inoculated immediately after preparation with 10 mg. of pressed baker's yeast/l. (Distillers Co. Ltd.).

It was convenient to grow the yeast in a Winchester quart bottle containing 2 l. of the medium. This was incubated for 40 hr. at 30° and during the incubation a vigorous stream of air (1·5–2 l./min.) was drawn through the medium. The air was previously passed through water at 30° to minimize evaporation of the medium. At the end of the incubation period the yeast was centrifuged, dehydrated with ethanol and refluxed for 30 min. with light petroleum to remove lipid material. The residue was filtered and washed once with ether. The yield of crude protein residue was consistently between 3·0 and 3·5 g./l. medium. After drying at 60–70° the residue was dust-like and was easily disturbed by slight air currents. The dry weight was therefore taken in a small flask (250 ml.) into which the still ether-damp residue was placed. The next stage of the isolation was carried out in this flask, thus avoiding pollution of the air with radioactive dust.

Preparation of radioactive L-*cystine*

Initial experiments showed, in agreement with Csonka (1935), that during the hydrolysis of yeast protein with hydrochloric acid there occurred considerable destruction of cystine. An attempt to block the breakdown of the liberated labelled L-cystine was made by conducting the hydrolysis in the presence of added L-cystine, but it was found that the recovery of cystine from the hydrolysate was low.

Bailey (1937) has obtained evidence that the destruction of cystine during the hydrolysis of plant proteins is associated with the presence of pentoses; these are presumably converted into furfural which then condenses with the amino-acids. As yeast protein is known to contain a considerable quantity of pentoses combined in nucleoproteins, the protein residue was extracted with hot trichloroacetic acid solution before the hydrolysis with hydrochloric acid. Schneider (1945) has shown that hot trichloroacetic acid solution quantitatively extracts the nucleic acids from animal tissues. This extraction of the yeast protein residue coupled with the addition of L-cystine enabled a satisfactory yield of labelled L-cystine to be obtained.

The lipid-free yeast residue from 2 l. of medium was extracted with 70 ml. of 5% (w/v) trichloroacetic acid solution for 15 min. at 90°, centrifuged, and washed once with cold 5% trichloroacetic acid solution. The damp residue was hydrolysed with 50 ml. boiling 5N-HCl in the presence of L-cystine (50 mg./g. yeast residue) which had been recrystallized from commercial samples. The time of hydrolysis was kept short (5·5 hr.), as experiments showed that the specific activity of the product was no higher after a longer hydrolysis, which at the same time would increase the degree of racemization (Hoffman & Gortner, 1922) and homocystine formation (Butz & Du Vigneaud, 1932). The hydrolysis was conducted in a fume chamber to remove any volatile sulphur compounds which might be released during the reaction (Grossfeld & Young-Yen, 1941). The hydrolysate was filtered from acid-insoluble humin and the filtrate boiled for a few min. with 200 mg. of activated charcoal (calcium phosphate-free). After removal of the charcoal the light-brown solution was evaporated to a viscous residue under reduced pressure. The residue was taken up in 50 ml. water and adjusted to pH 4·5 with 10N-NaOH solution. After standing for 24 hr. at 5° the crude cystine was filtered off and washed with water. The crude cystine was suspended in 60 ml. of warm water and HCl added until it had just dissolved. This solution was boiled with 40 mg. of activated charcoal until colourless, filtered and the cold filtrate adjusted to pH 4·5 with a saturated aqueous solution of sodium acetate. After standing 2 hr. at 0° the precipitated cystine was filtered off and re-precipitated from acid solution using NH_3 to neutralize the solution. Yield: approximately 50% based on the added L-cystine.

The L-cystine isolated as described consisted of the typical irregular and hexagonal plates of the precipitated compound. It gave a correct analysis for N and S, and $[\alpha]_D^{22°}$ in a typical experiment was −210·7° in N-HCl and did not change on recrystallization. The product was free from tyrosine when examined by chromatography and by the method of Arnow (1937).

The cystine had a counting rate of approximately 3×10^7 counts/min./m-mole when measured under a mica-window Geiger-Müller counting tube (mica, 1·7 mg. sq.cm.). The absence of traces of sulphur-containing contaminants of a high specific activity was demonstrated by the following observations. On twice precipitating at the isoelectric point and then crystallizing from a 50% (v/v) ethanol-water mixture, the specific activity of the cystine was unchanged. On conversion of the product to dibenzoylcystine, m.p. 180° (Gortner & Hoffman, 1921), the specific activity of the sulphur remained constant. Similarly, when thiazolidine-4-carboxylic acid, m.p. 196°, was prepared from the cystine (Rachelle, Reed, Kidwai, Ferger & Du Vigneaud, 1950) no change in the specific activity of the sulphur was observed. The cystine obeyed the constant-solubility test of Gutmann & Wood (1949) for the detection of radioactive impurities using water as the test solvent.

The absence of labelled homocystine from the product was tested for by dissolving excess DL-homocystine in a HCl solution of the product and precipitating the homocystine at pH 5·5. After thirteen such precipitations the specific activity of the homocystine was low enough to indicate that even if it were uncontaminated with cystine, less than 0·1% of the radioactivity of the cystine sample could be in the form of homocystine.

Isolation of labelled methionine

Radioactive methionine was isolated from the mother liquor of the cystine precipitation after the addition of L-methionine. Preliminary experiments showed that it was not advantageous to add the methionine before the hydrolysis of the yeast protein, nor was the specific activity of the isolated methionine increased by prolonging the hydrolysis to 18 hr. In an initial series of experiments the methionine was isolated from the hydrolysate by the method of Hill & Robson (1934), using their mercuric acetate reagent to precipitate the methionine directly from the hydrolysate. It was found that the product always contained traces of cystine and that this contamination was extremely difficult to prevent. Preliminary removal of the cystine from the hydrolysate by precipitation with cuprous oxide removed large quantities of the active methionine even after the hydrolysate had been boiled with zinc dust previous to the precipitation (see, Bailey, Chibnall, Rees & Williams, 1943; Beveridge & Lucas, 1944). An attempt was made to remove the cystine from a sulphuric acid hydrolysate with mercuric sulphate, but the subsequently isolated methionine was still impure, showing that the cystine precipitation was incomplete, although the reagent appears to precipitate cystine nearly quantitatively from a pure solution (Lavine, 1937). It became necessary, therefore, to prevent radioactive contamination by reducing the specific activity of the cystine remaining in the hydrolysate to negligible proportions by the addition of cystine before isolating the methionine (Williams & Dawson, 1952).

It was then found that when the method of Pirie (1932) was used for the isolation of the methionine the product was free from detectable amounts of cystine. Although in this method the precipitate formed with mercuric acetate is extracted with hot baryta the resulting racemization of the L-methionine appears to be very small. In the original method for the isolation of methionine from casein (Mueller, 1923) which involves several similar extractions with hot baryta, the product had a specific optical rotation very close to the correct value for L-methionine. The butanol extraction of the methionine from the hydrolysate used in Pirie's method probably leaves a considerable portion of the cystine in the acid layer (Hess & Sullivan, 1935). Moreover, the removal of barium ions with sulphuric acid during the methionine isolation would remove cystine possibly as a double salt with barium sulphate (Chibnall, Rees & Williams, 1943).

The mother liquor from the cystine precipitation was acidified with 5 ml. conc. HCl, and evaporated under reduced pressure to a viscous residue. The hydrochlorides of the amino-acids were dissolved in ethanol, and the solution was filtered from a residue of salt. The filtrate was evaporated *in vacuo* and the residue re-dissolved in 50 ml. of 0·7N-HCl. L-Methionine (Nutritional Biochemicals Corp.: Cleveland) was added; 100 mg./g. of yeast protein residue hydrolysed. The method of isolation then followed closely the procedure of Pirie (1932), with a number of minor modifications. The hydrolysate was extracted with 200 ml. *n*-butanol which had previously been saturated with aqueous N-HCl. After a double extraction with aqueous NaOH solution as described by Pirie (1932), the combined alkaline extracts were adjusted to pH 5 with HCl and evaporated under reduced pressure until free of butanol. Water was added to 250 ml. and saturated mercuric acetate in 3% (v/v) aqueous acetic acid was added until no further precipitation occurred. The precipitate was centrifuged and washed once with water, suspended in 50 ml. of water and added to 200 ml. of boiling 0·33N-$Ba(OH)_2$. The mixture was filtered hot, and the residue again extracted with 200 ml. of boiling 0·14N-$Ba(OH)_2$. The combined baryta extracts were freed from Ba ions with H_2SO_4 and the solution evaporated to dryness under reduced pressure. The residue was taken up in 10 ml. of hot water, and the methionine precipitated by the addition of a boiling solution of 5 g. $HgCl_2$ in 60 ml. water. After boiling for a few minutes the mixture was cooled, and stored at 0° for 2 hr. when the supernatant was decanted from the putty-like mercury complex. The latter was kneaded with a small quantity of ice-cold water, and then transferred to about 70 ml. warm water and decomposed with H_2S. The HgS was removed and thoroughly extracted with boiling water. The combined filtrate and washings were re-filtered and evaporated to dryness under reduced pressure. The residue was taken up in 5 ml. of ethanol and the methionine precipitated by adjusting to pH 5 with pyridine and standing at 0°. The precipitate was dissolved in 2 ml. of hot water, filtered and crystallized by adding 7 ml. of ethanol. Yield: approximately 30% of the added L-methionine, as shining plates giving a correct analysis for N and S.

Two-dimensional paper chromatography failed to reveal the presence of other amino-acids. The counting rate with the counter previously described was approximately 2×10^7 counts/min./m-mole. The specific activity of the methionine did not change on recrystallizing twice from 80% (v/v) ethanol. The specific activity of the sulphur remained constant on conversion of the methionine into the α-naphthyl isocyanate derivative, m.p. 187–188° (Mueller, 1923), this being recrystallized from ethanol-water. The absence of cystine in the methionine was shown by running 0·2 mg. of it on a one-dimensional paper chromatogram using a water-saturated phenol solvent, after which the dried chromatogram was sprayed with ninhydrin solution. The methionine was initially oxidized with H_2O_2 according to the method of Dent (1948). It was observed that when this amount of methionine was treated on the paper with

H_2O_2 for a prolonged period, oxidation to the sulphone was far from quantitative. Even when ammonium molybdate was also present during the oxidation, two main spots could always be seen on the chromatogram. Moreover, it was found that pure samples of synthetic methionine on chromatography gave a faint ninhydrin spot with a small R_F value indistinguishable from homocysteic acid. It is assumed that during the oxidation of methionine with H_2O_2 a small portion is demethylated and oxidized to homocysteic acid. No cysteic acid spot could be observed on the chromatogram of the labelled L-methionine (<0·5% cystine). The absence of homocystine from the product was shown by adding DL-homocystine to a sample of the methionine dissolved in dilute HCl and isolating the homocystine at the isoelectric point. After purification the homocystine was found to have negligible radioactivity (<0·2%).

DISCUSSION

The requirements of a satisfactory method for the preparation of a labelled amino-acid are that the procedure should be reasonably simple, the starting materials easily obtained, the product of a sufficiently high specific activity and free from impurities either inactive or active. The preparation may need to be repeated several times during an investigation especially when the tracer used decays relatively quickly. The methods described in this paper for the preparation of ^{35}S-labelled L-cystine and L-methionine have proved in practice to be very suitable for the intermittent preparation of these amino-acids. They require only apparatus and materials normally found in a small biological laboratory. During a series of preparations the unused radioactive sulphate ions in the culture medium can be recovered by precipitation with barium. The barium sulphate is then fused with eight equivalents of potassium carbonate which quantitatively converts it into potassium sulphate. The fusion mass is then extracted with water and the filtered extract, after neutralization with phosphoric acid, is added to the next culture medium which is adjusted accordingly.

Yeast was found to grow well in a medium 25 times more radioactive than the one described in the present series of experiments, with no diminution in the yield of crude yeast protein. The activity of the medium could probably be increased considerably further before the β-radiation affected the growth of the yeast cells.

Although Pirie's (1932) method for the isolation of methionine was better than that of Hill & Robson (1934) in that it gave a product free from detectable amounts of cystine it suffers from the disadvantage that a very small racemization of the amino-acid may occur during the isolation. In the intact animal and in isolated tissue preparations, methionine can be rapidly converted into cystine (Tarver & Schmidt, 1939; Melchior & Tarver, 1947), so that in animal experiments a trace of cystine in labelled methionine is not likely to be of great significance.

SUMMARY

1. A simple biosynthetic method is described for preparing L-cystine and L-methionine labelled with radioactive sulphur (^{35}S).

2. Baker's yeast was grown in a synthetic medium containing labelled sulphate ions, and the amino-acids were isolated from the yeast proteins.

3. The amino-acids isolated had high specific activities.

4. No impurities, either inactive or radioactive, were detected in the isolated products.

The authors are very grateful to Dr D. Richter for his continued interest in this work, and thank the Rockefeller Foundation and University of London for apparatus grants.

REFERENCES

Arnow, L. E. (1937). *J. biol. Chem.* **118**, 531.
Beveridge, J. M. R. & Lucas, C. C. (1944). *Biochem. J.* **38**, 88.
Bailey, K. (1937). *Biochem. J.* **31**, 1396.
Bailey, K., Chibnall, A. C., Rees, M. W. & Williams, E. F. (1943). *Biochem. J.* **37**, 360.
Butz, L. W. & Du Vigneaud, V. (1932). *J. biol. Chem.* **99**, 135.
Camien, M. N., Malin, R. B. & Dunn, M. S. (1951). *Arch. Biochem.* **30**, 62
Chibnall, A. C., Rees, M. W. & Williams, E. F. (1943). *Biochem. J.* **37**, 372.
Csonka, F. A. (1935). *J. biol. Chem.* **109**, 703.
Dent, C. E. (1948). *Biochem. J.* **43**, 169.
Gortner, R. A. & Hoffman, W. F. (1921). *J. Amer. chem. Soc.* **43**, 2199.
Graham, C. E., Hier, S. W., Waitkoff, H. K., Saper, S. M., Bibler, W. G. & Pentz, E. I. (1950). *J. biol. Chem.* **185**, 97.
Grossfeld, J. & Young-Yen, M. H. (1941). *Z. Untersuch. Lebensmitt.* **82**, 437.
Gutmann, R. H. & Wood J. L. (1949). *Science*, **110**, 662.
Hess, W. C. & Sullivan, M. X. (1935). *J. biol. Chem.* **108**, 195.
Hill, E. M. & Robson, W. (1934). *Biochem. J.* **28**, 1008.
Hoffman, W. F. & Gortner, R. A. (1922). *J. Amer. chem. Soc.* **44**, 341.
Lavine, T. F. (1937). *J. biol. Chem.* **117**, 309.
Melchior, J. & Tarver, H. (1947). *Arch. Biochem.* **12**, 309.
Mueller, J. H. (1923). *J. biol. Chem.* **56**, 157.
Neuberger, A. (1948). *Biochem. Soc. Symp.* 1, p. 20.
Olsen, B. H. & Johnson, M. J. (1949). *J. Bact.* **57**, 235.
Pirie, N. W. (1932). *Biochem. J.* **26**, 1270.
Rachelle, J. R., Reed, L. J., Kidwai, A. R., Ferger, F. & Du Vigneaud, V. (1950). *J. biol. Chem.* **185**, 817.
Schneider, W. C. (1945). *J. biol. Chem.* **161**, 293.
Schultz, A. S. & McManus, D. K. (1950). *Arch. Biochem.* **25**, 401.
Sugata, H. & Koch, F. (1935). *Plant Physiol.* **1**, 337.
Tarver, H. & Schmidt, C. L. A. (1939). *J. biol. Chem.* **130**, 67.
Williams, R. B. & Dawson, R. M. C. (1952). *Biochem. J.* **50**, xxi.
Woodward, G. E. (1951). *J. Franklin Inst.* **251**, 557.

The Direct Isolation of Arginine and Lysine Picrates from Gelatin Hydrolysates

By W. ROBSON and A. S. M. SELIM
Department of Physiology and Biochemistry, King's College, Strand, London, W.C. 2

(*Received* 1 *April* 1952)

The original process of Kossel & Kutscher (1900) of isolating arginine and lysine as their respective silver and picric acid salts from the phosphotungstic acid precipitates of protein hydrolysates is costly and tedious. These disadvantages are not entirely overcome by the electrodialysis method of Foster & Schmidt (1926). The direct preparation of arginine by the flavianic acid method (Kossel & Gross, 1924) and of lysine as picrate (Rice, 1939) yields products which require further purification (cf. Cox, 1928; Vickery, 1940; Heathcote, 1949).

In the method now described for the isolation of arginine and lysine, use has been made of the low solubilities of their picrates in water, arginine picrate about 0·5 g./100 ml. at 16° (Abderhalden, 1911) and lysine picrate about 0·54 g./100 ml. at 21° (Lawrow, 1899). The pH of a saturated aqueous solution of the former picrate, determined electrometrically at room temperature, is between 6·5 and 6·8, and of the latter between 5·0 and 5·4. When a quantity of picric acid, equivalent to the arginine present, was added to a concentrated gelatin hydrolysate (pH 8), previously freed from the sulphuric acid used for hydrolysis, no precipitate was formed even when the mixture was kept at 0° for several days. Adjustment of the pH of the mixture, now approximately 5·0, to 6·5–7·0 by the addition of ammonium hydroxide led, on rubbing the sides of the vessel with a glass rod, to the separation of the typical long bright thread-like crystals of arginine picrate. After a few hours the mixture was semi-solid. Collected at the pump and washed with ice-cold water, the compound, on recrystallization from boiling water, yielded a product of excellent melting point and free from histidine, lysine and ammonia. Decomposition of the picrate so obtained was easily effected by hot hydrochloric acid. From the picric acid-free solution arginine monohydrochloride has been obtained in amounts corresponding to 90% of the total amino-acid present in gelatin.

The further addition of picric acid to the solution from which the arginine picrate was removed, followed by the adjustment of the pH of the mixture to 5, led to the separation of lysine picrate in amounts corresponding to approximately 60% of the theory.

EXPERIMENTAL

Gelatin 'Spa' (600 g.; N, 17·17; moisture, 12·8%) was added to 2·5 l. of 8N-H_2SO_4 and the protein brought completely into solution by heating. When the protein had dissolved the mixture was refluxed for 30 hr. The acid hydrolysate was diluted with twice its own volume of water and neutralized to litmus with a suspension of $Ca(OH)_2$ in water. The $CaSO_4$ precipitate, which settled quickly, was removed at the pump, extracted twice with boiling water and the mixture again filtered. The filtrate and washings were combined and any SO_4 ions remaining in solution removed with $Ba(OH)_2$. Ca ions, still in solution, were removed with oxalic acid; the small precipitate in each case being collected and washed on the filter with boiling water. The hydrolysate freed from oxalate, SO_4, Ca and Ba ions had a pH about 8. It was then concentrated *in vacuo* to 2 l.

Isolation of arginine picrate. An estimation by the colorimetric procedure of Brand & Kassell (1942) showed that the solution contained approximately 44 g. of arginine. Solid picric acid (64 g., 10% in excess of the amount actually required to combine with all the arginine present) having been added, the mixture was heated with stirring until all the reagent was dissolved. The solution was cooled to room temperature and concentrated ammonia added with stirring until the pH was between 6·5 and 7. The solution became slightly turbid due to the precipitation of some ammonium picrate. After re-solution of these crystals, by heating, the mixture was cooled to room temperature under the tap. Long yellow needles soon commenced to form, first at the surface, and spread throughout the solution, until after about 1 hr. the whole solution had turned to a semi-solid mass of crystals. After 12 hr. at 0° the picrate was collected at the pump, sucked dry and then washed thrice with 100 ml. of ice-cold water. The product was then recrystallized from about 500 ml. of boiling water. After chilling the solution for 12 hr., the arginine picrate was again collected, washed first with a little ice-cold water, then with absolute ethanol and finally dried in the oven at 110° for several hours. The mother liquor from the recrystallized product and the washings were added to the original filtrate which was treated as shown below for the isolation of lysine picrate. The yield of recrystallized arginine picrate was 95 g., m.p.

217–218° (Vickery & Leavenworth, 1927). The product gave a negative Pauly reaction for histidine.

Arginine picrate contains 2 mol. of water of crystallization, which are lost at 110°. (Found: water of crystallization, 8·21. Calc. for $C_6H_{14}O_2N_4.C_6H_3O_7N_3.2H_2O$, 8·20%. Found: C, 35·9; H, 4·1; N, 23·4. Calc. for $C_6H_{14}O_2N_4.C_6H_3O_7N_3$: C, 35·73; H, 4·21; N, 24·29%.)

Preparation of arginine monohydrochloride. The arginine picrate was decomposed by hot HCl. The picric acid was removed from the mixture by extracting three times with hot benzene. The resulting solution was concentrated *in vacuo* to a thick syrup. This was taken up in hot ethanol and concentrated ammonia added drop by drop with stirring until the arginine monohydrochloride started to crystallize. After 12 hr. in the ice box the product was collected and recrystallized by dissolving it in the minimum amount of hot water and precipitating it with ethanol. The recrystallized arginine monohydrochloride melted at 216–217° (Cox, 1928).

Isolation of lysine picrate. The solution remaining after the removal of arginine picrate was acidified with H_2SO_4 and shaken with hot benzene to remove the picric acid. $Ca(OH)_2$ in slight excess of the H_2SO_4 present was added, the $CaSO_4$ removed, washed and the free ammonia removed by partial concentration of the filtrate *in vacuo*. SO_4 and Ca ions were exactly removed in the manner previously described, and the solution was concentrated *in vacuo* to about 500 ml. and allowed to stand overnight in the refrigerator. The mono-amino-acids which had separated were removed and washed several times with ice-cold water; the combined filtrate and washings had a volume of about 800 ml. This was treated with 48 g. of picric acid, heated until all the solid had dissolved and then cooled. The pH of the mixture was adjusted to 5 by the addition of 40% (w/v) NaOH. After 48 hr. in the refrigerator the mixture was filtered, the precipitated picrate collected, washed twice with ice-cold water, and recrystallized from boiling water. The picrate was collected, washed with a little water, then with ethanol and finally dried in the oven at 110°. The dried product weighed 32 g. and when heated decomposed at 261° with the characteristic explosion of pure lysine picrate.

Preparation of lysine monohydrochloride. According to the usual procedure the picrate was decomposed with hot HCl, the picric acid extracted with benzene, and the resulting solution concentrated *in vacuo* to a syrup. This was dissolved in the minimum amount of hot ethanol and pyridine was added gradually until the lysine monohydrochloride commenced to separate. The crude product was recrystallized from aqueous ethanol. The monohydrochloride had a melting point of 258–261° which was not depressed on admixture with an authentic specimen (Rice, 1949).

SUMMARY

1. Procedures for the isolation of arginine picrate and lysine picrate from gelatin hydrolysates are described. These picrates form convenient sources for the preparation of arginine monohydrochloride and lysine monohydrochloride in good yield and purity.

2. The method possesses advantages over the flavianic acid method in that it is economical in time and reagents. Picric acid is cheap, is used only in approximately stoicheiometric proportions and, finally, is much easier to recover than flavianic acid.

REFERENCES

Abderhalden, E. (1911). *Biochem. Handlexikon*, **4**, 631.
Brand, E. & Kassell, B. (1942). *J. biol. Chem.* **145**, 359.
Cox, C. J. (1928). *J. biol. Chem.* **78**, 475.
Foster, G. L. & Schmidt, C. L. A. (1926). *J. Amer. chem. Soc.* **48**, 1709.
Heathcote, J. G. (1949). Thesis, University of London.
Kossel, A. & Gross, R. E. (1924). *Hoppe-Seyl. Z.* **135**, 167.
Kossel, A. & Kutscher, F. (1900). *Hoppe-Seyl. Z.* **31**, 165.
Lawrow, D. (1899). *Hoppe-Seyl. Z.* **28**, 388.
Rice, E. E. (1939). *J. biol. Chem.* **131**, 1.
Rice, E. E. (1949). *Biochem. Prep.* **1**, 63.
Vickery, H. B. (1940). *J. biol. Chem.* **132**, 325.
Vickery, H. B. & Leavenworth, C. S. (1927). *J. biol. Chem.* **72**, 403.

Effect of Choline Deficiency on the Fatty Acids of Liver and Body Lipids in the Rat

By C. SIVA RAMAN
National Chemical Laboratory of India, Poona

(*Received* 16 *April* 1952)

Changes in the nature and amounts of tissue lipids are of special significance in elucidating the mechanism by which choline prevents liver-fat deposition. Numerous investigations have been concerned mainly with changes in the relative proportions of the various lipid fractions. Liver-fat accumulation during choline deficiency in the rat is characterized almost exclusively by an increase in glycerides with a proportionate rise in cholesterol content, mostly in the form of cholesteryl esters (Best, Lucas, Patterson & Ridout, 1946). The total phospholipin content of the liver is generally not altered, although a decrease in the proportion of choline-containing phospholipins has been observed by several authors (Stetten & Grail, 1942; Artom & Fishman, 1943; Fishman & Artom, 1944, 1946; Campbell, Olley & Blewett, 1949).

Much less attention has been given to the study of variations in the nature of the fatty acids of liver lipids. Channon & Wilkinson (1936) investigated the effect of the chemical nature of food fat on the nature of the fatty acids deposited in the liver of rats when various fats were fed for 2 weeks at a 40 % level with a choline-deficient, 5 % casein diet. The total liver lipids were fractionated into phospholipins and glycerides and the fatty acids of each fraction separated into solid and liquid acids by the Twitchell (1921) procedure. The results indicated that in the glyceride fraction the proportion and mean molecular weights of the solid and liquid acids and the iodine numbers of the liquid acids were influenced by the nature of the food fats, while in the phospholipins only the molecular weights of the fatty acids varied with the type of dietary fat. Channon, Jenkins & Smith (1937) also investigated in detail the total carcass fatty acids in these animals. The saturated acids of carcass lipids, like the solid acids of liver glycerides, were influenced by the composition of the dietary fat. There was no apparent relationship between the total fatty acid content of the carcasses and the percentage of lipids in the livers.

The present paper deals with the effect of choline on the amount and nature of the fatty acids of the phospholipin and glyceride fractions of liver and carcass of rats maintained on diets free from unsaturated fatty acids. In the light of current views on the function of choline it appeared probable that the exclusion of unsaturated acids would give a clearer indication of the changes taking place in choline deficiency. With the object of studying the effect of choline under conditions wherein fat deposition is due on the one hand to assimilation from dietary fat and on the other to synthesis from carbohydrate, two different basal diets were used, one containing 30 % of fully saturated glycerides, while the other was fat-free.

Table 1. *Percentage composition of basal diets*

Diet	Casein*	Fully saturated glycerides	Glucose	Cellulose†	Salt‡ mixture
High-fat	5	30	61	0	4
Fat-free	5	0	89	2	4

* Light white (British Drug Houses Ltd.) extracted with ethanol.
† Prepared from filter paper (Whatman no. 1) pulp.
‡ Steenbock-Nelson salts 40 (Steenbock & Nelson, 1923).

EXPERIMENTAL

The choline-free basal diets were made up as shown in Table 1.

The fully saturated glycerides (iodine value, I.V., 0) used in the diets were prepared from buffalo depot fat. The fat (after removal of free fatty acids) was dissolved in lots of 500 g. in acetone (5 ml./g. fat), left overnight at room temperature (approximately 30°) and the separated fat obtained by filtration. The insoluble portion was redissolved in acetone (3 ml./g. fat) and left overnight at room temperature. The treatment resulted in the deposition of a crop of about 150 g. with I.V. of about 13. The final crystalline fraction was oxidized in batches of 100 g. for preparation of fully saturated glycerides according to the method of Hilditch & Lea (1927) and the neutral, completely saturated

glycerides separated from the acidic oxidation products. The preliminary crystallizations from acetone ensured complete oxidation in one operation.

Young albino rats weighing 110–150 g. were placed in individual cages over wide-mesh screening in groups of eight, animals of the same sex being distributed evenly between the groups. Each animal received in addition to the basal diets described above, the following vitamin supplements: thiamine hydrochloride, 25 μg.; riboflavin, 20 μg.; pyridoxine hydrochloride, 25 μg.; nicotinic acid, 100 μg.; vitamin A, approximately 150 i.u. and vitamin D, approximately 25 i.u. The water-soluble vitamins were fed by tube. Vitamins A and D were prepared as a fat-free concentrate by saponification and ether extraction of a commercial preparation; the ether was sprayed on to the food and the solvent removed *in vacuo*. The animals on the choline-containing diets received in addition 23 mg. choline chloride, corresponding to 20 mg. of choline per rat per day. The diets were fed *ad lib.* and the daily food intakes recorded. After 30 days on the experimental diets the animals were weighed, killed by stunning and their liver and carcass lipids analysed.

Analysis of liver and carcass lipids

Extraction of liver and carcass lipids. The livers were removed immediately after killing, weighed and ground with sand under ethanol. The pooled livers from each group were extracted in the Soxhlet with ethanol and finally with $CHCl_3$. The ethanol and $CHCl_3$ extracts were separately made up to volume and samples combined for analysis.

Each carcass, after removal of the liver, was worked up immediately as follows: the head, tail and gastro-intestinal tract were removed and the remaining carcass weighed, cut into small pieces and stirred with about 3 vol. of hot acetone. The carcasses from each group were pooled, dehydrated further by boiling with two more lots of acetone and the residue extracted for 3 hr. in the Soxhlet with fresh acetone. The dehydrated material was then sucked dry at the pump, the residue ground to a powder and re-extracted in the Soxhlet with ethanol and the extraction completed with $CHCl_3$. The solvents were separately made up to volume and samples from the various extracts combined for analysis.

Separation of phospholipins. The phospholipins were separated from neutral fat ('glycerides') and unsaponifiable matter by precipitation with acetone and $MgCl_2$ (Sinclair, 1930). Portions of the various extracts corresponding to a total of about 200 mg. phospholipin fatty acids (approximately 12 g. liver and 35 g. of carcass) were mixed and taken to dryness at 40–45° under reduced pressure in an atmosphere of N_2. The residue was extracted with ether (about 60 ml. with washings) and poured into 420 ml. acetone. To facilitate complete precipitation of the phospholipins, a saturated solution of $MgCl_2.6H_2O$ in ethanol (2 ml.) was added with stirring. After centrifuging for 5 min. at low speed the supernatant acetone-ether was poured out, the precipitate stirred with 100 ml. of fresh acetone, centrifuged again for 1 min. and the extracts combined. The residue was stirred into 40 ml. ether and the phospholipin fraction precipitated and washed as before. To verify the complete absence of glycerides from the phospholipin fraction a sample of the phospholipins obtained as described was in certain cases precipitated a third time. The analysis of phospholipin fatty acids resulted in almost identical values for solid acid content whether two or three precipitations had been made.

Analysis of phospholipin and glyceride fatty acids. The fatty acids were obtained from the phospholipin and glyceride fractions according to methods described by Sinclair (1930, 1940), the separation and liberation of the acids being carried out in an atmosphere of N_2. Approximately 200 mg. fatty acids from both the fractions were analysed by the Twitchell procedure as modified by Sinclair (1940), a Hirsch funnel equipped with an outer hot-water jacket being used, however, for filtering the ethanolic solutions of the lead salts as recommended by Horning & Eckstein (1946).

Iodine numbers of fatty acid fractions were determined according to Yasuda (1931), and the mean molecular weights by titration with 0·05 *N*-sodium ethoxide in ethanol (Fowweather, 1926).

Table 2. *Average food intakes and weight records*

	High-fat diet		Fat-free diet	
	Basal	Basal + choline	Basal	Basal + choline
Average initial body weight (g.)	115	116	141	141
Average final body weight (g.)	99	106	137	140
Average food intake (g./rat/day)	5	5	7	7
Liver (average % body weight)	4·07	2·93	3·37	2·99

Table 3. *Characteristics of liver lipids of rats*

((*a*) Values expressed as percentage fresh liver weight; (*b*) values expressed as mg./100 g. body weight.)

	High-fat diet				Fat-free diet			
	Basal		Basal + choline		Basal		Basal + choline	
	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)
Phospholipin fatty acids	1·198	48·77	1·698	49·72	1·572	52·91	1·833	54·77
Glyceride fatty acids	22·13	901·8	0·982	28·72	10·78	363·1	2·709	80·95
Unsaponifiable matter	1·227	50·00	0·146	4·27	0·514	17·32	0·356	10·65

RESULTS

The food intakes and weight records of the various groups are set out in Table 2.

Rats on the high-fat diets lost weight, the loss being more pronounced on the choline-free diet, whereas animals on the fat-free diets maintained

Table 4. *Carcass lipids*

(Values expressed as percentage of carcass weight.)

	High-fat diet		Fat-free diet	
	Basal	Basal + choline	Basal	Basal + choline
Phospholipin fatty acids	0·4490	0·4742	0·5152	0·5344
Glyceride fatty acids	1·517	2·793	11·87	12·77
Unsaponifiable matter	0·526	0·513	0·581	0·564

Table 5. *Effect of diet on phospholipin fatty acids of liver and carcass*

	High-fat diet		Fat-free diet		
	Basal	Basal + choline	Basal	Basal + choline	Stock
LIVER					
Solid acids:					
%	44·3	31·6	38·7	30·9	33·0
I.V.	3	4	4	2	6
Mol.wt.	273	270	282	282	—
Liquid acids:					
%	55·7	68·4	61·3	69·1	67·0
I.V.	266	228	223	202	218
Mol.wt.	338	330	326	328	—
CARCASS					
Solid acids:					
%	33·2	34·3	29·8	30·5	—
I.V.	3	6	3	5	—
Mol.wt.	266	270	265	265	—
Liquid acids:					
%	66·8	65·7	70·2	69·5	—
I.V.	178	179	147	144	—
Mol.wt.	341	334	320	340	—

Table 6. *Effect of diet on glyceride fatty acids of liver and carcass*

	High-fat diet		Fat-free diet		
	Basal	Basal + choline	Basal	Basal + choline	Stock
LIVER					
Solid acids:					
%	29·4	28·5	28·3	24·0	24·8
I.V.	5	8	8	6	9
Mol.wt.	269	271	272	272	—
Liquid acids:					
%	70·6	71·5	71·7	76·0	75·2
I.V.	108	124	99	95	107
Mol.wt.	298	295	289	291	—
CARCASS					
Solid acids:					
%	42·7	37·6	34·1	33·8	—
I.V.	8	6	6	7	—
Mol.wt.	289	290	264	262	—
Liquid acids:					
%	57·3	62·4	65·9	66·2	—
I.V.	98	99	94	90	—
Mol.wt.	295	290	275	278	—

their body weights during the period of the experiment. The average food intakes of animals in all the groups, however, were approximately the same when calculated for the 100 g. rat.

The composition of the liver lipids is shown in Table 3 and of the carcass lipids in Table 4.

In animals on the high-fat diets an inverse relationship is apparent between the glyceride fatty acid contents of liver and carcass, choline reducing the glyceride content from 22·13 to 0·982 % in the liver and increasing it from 1·517 to 2·793 % in the body depots. The values indicate that under the experimental conditions choline is necessary for the normal distribution of fat between the liver and the body. On the fat-free diets, the glyceride acid content of carcass is unaffected by choline, the values being 11·87 % without and 12·77 % with the supplement. Choline has apparently no effect on deposition of fat in body depots when fat is derived from dietary carbohydrate. On both types of diet

choline is without influence on the amount of phospholipin fatty acids of liver and carcass and the unsaponifiable matter of carcass.

Tables 5 and 6 record the variations in the nature of the fatty acids of the phospholipin and glyceride fractions. Values for liver fatty acids from a group of stock animals of approximately 100 g. body weight are included for comparison.

DISCUSSION

The results obtained are best discussed under two separate heads.

Effect of choline on liver lipids. As was expected, choline deficiency caused excessive deposition of liver fat in animals on the high-fat as well as on the fat-free diets, the liver-fat content (total fatty acids + unsaponifiable matter) being 24·5 % in the former and 12·9 % in the latter. The addition of choline to the diet reduced the values to 2·8 and 4·9 % respectively, indicating that the moderate liver-fat accumulation which takes place on a fat-free diet is possibly less susceptible to the corrective action of choline.

Interesting changes were observed in the nature of phospholipin fatty acids of liver during choline deficiency. In the absence of choline the proportion of solid acids increased from 31·6 to 44·3 % on the high-fat diet and from 30·9 to 38·7 % on the fat-free diet. The degree of unsaturation of the liquid fatty acids of liver phospholipin was also influenced by dietary choline, the iodine number of the liquid acids being considerably higher in choline deficiency (Table 5).

Although the effect of choline is most marked on the glyceride content of the liver it is apparent from the results described in Table 6 that choline does not have any appreciable influence on the fatty acid composition of liver glycerides. The percentages of the solid fatty acid in liver glycerides are approximately the same in animals on high-fat diets, the values being 28·5 % with choline and 29·4 % without choline and only slightly different from each other in those on the fat-free ration, the corresponding values being 24·0 and 28·3 % respectively. As between the high-fat and fat-free diets, the unsaturation of the liquid acids of liver glycerides is greater on the former diet, the iodine numbers in the two cases being 108 and 99. Supplementation with choline raises the former value to 124, but appears to have no significant effect on the latter.

Effect of choline on body lipids. It is of interest to note the high lipid content of the carcass of animals on the fat-free diet as compared to those on the high-fat ration (Table 4). This is evidently due to synthesis of fat from dietary carbohydrate as is borne out by the low molecular weights of the glyceride fatty acids (Table 6). Spadola & Ellis (1936) and Longenecker (1939) observed a high proportion (40–45 %), of low molecular weight fatty acids, of the C_{16} series in particular, in the body fat of animals maintained on a low-fat, high-carbohydrate ration and came to the conclusion that such high proportions of the lower fatty acids are characteristic of fat synthesized from carbohydrate. The results obtained in the present investigation would indicate that choline has no role in this synthetic process since animals on the basal fat-free diet have the same high carcass-glyceride content and the characteristic low mean molecular weight of the glyceride acids as the corresponding choline-supplemented group.

In contrast to the observations noted above on the effect of choline on the fat content and phospholipin composition of liver, choline administration produces little or no effect on either of the lipid fractions of carcass fat.

SUMMARY

1. The effect of choline on the fatty acids of liver and carcass lipids has been investigated on rats maintained on two different basal diets, one containing 30 % of fully saturated glycerides, the other being fat-free.

2. Animals on the high-fat diets lost in body weight, the loss being more pronounced in the absence of choline. Choline effectively prevented fatty livers and caused a slight increase in the low carcass-fat content of these animals.

3. Rats on the fat-free diets maintained their body weight and had a carcass-fat content markedly higher than animals on the high-fat diets. Fat accumulation in the liver in the absence of choline was moderate and at the same time not completely prevented by addition of choline.

4. Choline had a marked effect on the nature of liver phospholipin fatty acids: (i) the proportion of solid fatty acids in phospholipin fatty acids of liver was considerably higher in choline deficiency both on the high-fat diet and fat-free ration, (ii) iodine numbers of the liquid acids of liver phospholipin were higher in the choline-deficient groups.

5. No changes in the nature of the fatty acids of liver and carcass glycerides or of carcass phospholipins were noticed. In the case of the liver glycerides the effect of choline was apparent only on the content and not on the fatty acid composition.

6. The mean molecular weights of the liquid acids of carcass glycerides in animals on fat-free diets were characteristic of body fat derived from dietary carbohydrate. Choline, evidently, has no role in this synthetic process, since animals on such diets had the same high carcass-glyceride content both in the presence and absence of choline.

I wish to thank Prof. M. Damodaran for his interest and advice during this investigation.

REFERENCES

Artom, C. & Fishman, W. H. (1943). *J. biol. Chem.* **148**, 423.
Best, C. H., Lucas, C. C., Patterson, J. M. & Ridout, J. H. (1946). *Biochem. J.* **40**, 368.
Campbell, I. G., Olley, J. & Blewett, M. (1949). *Biochem. J.* **45**, 105.
Channon, H. J., Jenkins, G. N. & Smith, J. A. B. (1937). *Biochem. J.* **31**, 41.
Channon, H. J. & Wilkinson, H. (1936). *Biochem. J.* **30**, 1033.
Fishman, W. H. & Artom, C. (1944). *J. biol. Chem.* **154**, 109.
Fishman, W. H. & Artom, C. (1946). *J. biol. Chem.* **164**, 307.
Fowweather, F. S. (1926). *Brit. J. exp. Path.* **7**, 7.
Hilditch, T. P. & Lea, C. H. (1927). *J. chem. Soc.* p. 3106.
Horning, M. G. & Eckstein, H. C. (1946). *J. biol. Chem.* **166**, 711.
Longenecker, H. E. (1939). *J. biol. Chem.* **128**, 645.
Sinclair, R. G. (1930). *J. biol. Chem.* **88**, 575.
Sinclair, R. G. (1940). *J. biol. Chem.* **134**, 71.
Spadola, J. M. & Ellis, N. R. (1936). *J. biol. Chem.* **113**, 205.
Steenbock, H. & Nelson, E. M. (1923). *J. biol. Chem.* **56**, 355.
Stetten, D. & Grail, G. F. (1942). *J. biol. Chem.* **144**, 175.
Twitchell, E. (1921). *J. industr. Engng Chem.* **13**, 806.
Yasuda, M. (1931). *J. biol. Chem.* **94**, 401.

The Effect of Light on Xanthine Oxidase Activity

By S. M. SIEGEL* and R. L. WEINTRAUB
Chemical Corps Biological Laboratories, Frederick, Maryland, U.S.A.

(*Received* 13 *March* 1952)

It has been postulated, on the basis of studies of activation by light, that a flavin enzyme is involved in the indolylacetic acid oxidase system (Galston, 1950; Galston & Baker, 1951). The only report of light activation of a flavin enzyme appears to be that of Bernheim & Dixon (1928); working with a crude preparation of xanthine oxidase they found either activation or inactivation according to the conditions of the particular experiment. In a later study of the same enzyme only an inactivating effect of light was observed (Toyama, 1933). It has seemed important, therefore, to re-investigate the effect of light on xanthine oxidase.

EXPERIMENTAL

Enzyme. Milk from which casein had been removed after precipitation by rennin served as the source of enzyme in the majority of the experiments. In a few instances comparative tests were made with whole milk.

Substrate. Xanthine or hypoxanthine was employed as substrate. Preliminary studies indicated that maximal activity was obtained with 5×10^{-5} M-substrate.

Hydrogen acceptor. Methylene blue (MB), at 1×10^{-5} M, or nitrate, at 5×10^{-3} M, was used as hydrogen acceptor. The measurements were carried out in 0·05 M-phosphate buffer of pH 8·8 in the case of MB and of pH 5·5 with nitrate.

Measurement of enzyme activity. With MB, enzyme activity was measured by following spectrophotometrically with an Evelyn photoelectric colorimeter (620 mμ. filter) the time course of decolorization of the dye in evacuated Thunberg tubes. Activity was conveniently expressed as the reciprocal of the time required for 25 or 50% reduction. To measure the rate of nitrate reduction, a series of identical tubes was prepared and the nitrite determined successively in individual tubes, using a modification of the method of Parker (1949). The reaction rates were measured at 25° with MB and at 37° with nitrate.

Irradiation. The solution of enzyme in buffer, with or without hydrogen acceptor, was irradiated in open tubes immersed in a water bath at 25–27°, frosted tungsten filament lamps serving as the sources. The tubes were illuminated from two opposite sides and were shaken frequently. The non-irradiated control tubes, which were wrapped with aluminium foil, were manipulated concurrently in exactly the same way. At the highest intensities and longest exposures, the temperature of the illuminated solutions containing MB rose as much as 8° above that of the dark controls; tests showed that holding the enzyme solution in darkness at the elevated temperature did not affect its activity subsequently at 25°.

Procedure. Immediately after irradiation the tubes were closed and evacuated, the zero time transmission determined, the substrate tipped in, and readings commenced.

RESULTS

Light was found to influence the activity of xanthine oxidase only if an added photoreceptive pigment was present during the irradiation. Fig. 1 illustrates a typical experiment with MB.

Riboflavin also was found capable of serving as a photoreceptor in mediating light inactivation of xanthine oxidase. A comparison of the effectiveness of the two pigments was obtained as follows. Various amounts of riboflavin were added to portions of enzyme solution and MB to others. After irradiation at 1800 ft.c. for 5 min., MB was added to the riboflavin samples and the activities of all were measured in the usual manner. Riboflavin does not

* Present address: Department of Botany, University of Chicago, Chicago, Illinois.

interfere with the spectrophotometric estimation of MB at 620 mμ. In both cases the degree of inactivation was roughly proportional to the initial concentration of photoreceptor. For equal inactivation, about 2·5 times as great a concentration of riboflavin as of MB was required. It should be pointed out, however, that because of the much higher molecular extinction of MB, the total energy absorbed was about 11·5 times as great as by the riboflavin. This factor was approximated by measuring the areas under the curves which were obtained by multiplying the molar absorption spectra, in the region 350–750 mμ. (determined with a Beckman model DU spectrophotometer), by the emission spectrum of the source, as given by Taylor (1934).

By using nitrate as hydrogen acceptor, the dye may be eliminated entirely. In this case, exposure either to bright sunlight or to 'daylight' fluorescent radiation of about 500 ft.c. was without effect on the activity of the enzyme.

Figs. 2 and 3 present the results of experiments to ascertain whether activation might result from shorter exposure or lower intensity.

As these experiments gave no evidence of activation of the enzyme, in complete disagreement with those of Bernheim & Dixon (1928), a number of variations were tried in an attempt to discover the cause of the discrepancy. Inactivations entirely similar to the foregoing were obtained also with whole milk in place of the decaseinated preparation, with hypoxanthine rather than xanthine as substrate, at pH 6·8 and 7·4, and with various intervals of darkness interposed between the irradiation and measurement of activity.

Bernheim & Dixon (1928) believed that the effect of light was due to the formation of hydrogen

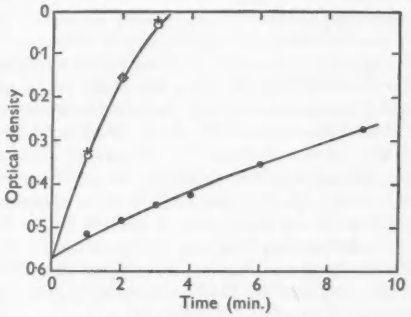


Fig. 1. Influence of 30 min. irradiation at 1800 ft.c. on xanthine oxidase activity. Substrate, xanthine. Hydrogen acceptor, methylene blue. ○, non-irradiated; +, irradiated without methylene blue; ●, irradiated with methylene blue.

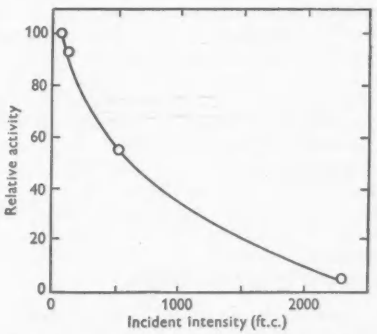


Fig. 2. Effect of light intensity on inactivation of xanthine oxidase. Exposure time, 30 min. Substrate, xanthine. Hydrogen acceptor, methylene blue.

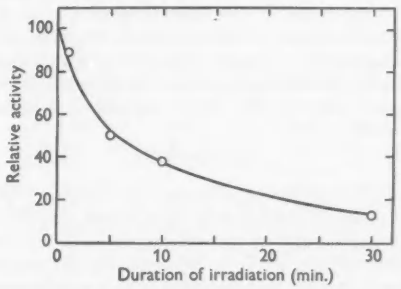


Fig. 3. Effect of duration of irradiation of xanthine oxidase. Intensity, 1800 ft.c. Substrate, xanthine. Hydrogen acceptor, methylene blue.

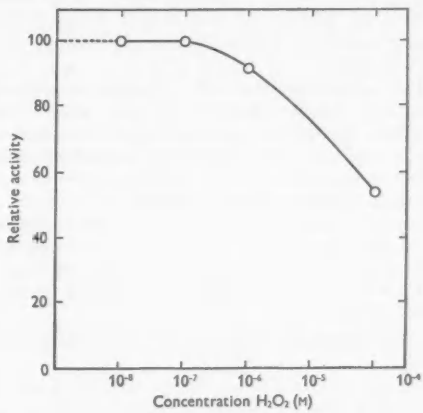


Fig. 4. Effect of added hydrogen peroxide on activity of xanthine oxidase. Substrate, xanthine. Hydrogen acceptor, methylene blue.

peroxide and presented data showing activation of xanthine oxidase on addition of small quantities of this substance in the dark. As shown in Fig. 4, we have been unable to reproduce this result either. It might be supposed that catalase, if present in the xanthine oxidase preparation, would tend to prevent activation either by hydrogen peroxide or by light, and indeed Bernheim & Dixon (1928) ascribed to the presence of catalase their failure to obtain activation with some of the milk samples tested. This explanation does not seem tenable, however, as we have obtained virtually identical inactivations, by light, of a xanthine oxidase preparation having high catalase activity and of one devoid of detectable catalase.

DISCUSSION

From the discrepancy between the foregoing results and those of Bernheim & Dixon (1928) it is evident that various crude preparations of xanthine oxidase may be affected entirely differently by illumination, whence it may be concluded that the influence of light is not directly upon the enzyme itself. As activations were never encountered in the present experiments, this indirect effect was not amenable to study, and no definitive explanation of the apparent activation observed by Bernheim & Dixon (1928) can be presented. However, one or two suggestions may be noted.

It is conceivable that light or hydrogen peroxide may diminish the effect of some inhibitor of the enzyme. In this connexion it is of interest that Kalckar & Klenow (1948) have shown that folic acid, which is known to occur in some samples of milk (Hodson, 1949), markedly inhibits the activity of xanthine oxidase. The inhibition was later ascribed to a decomposition product of folic acid and was shown to be relieved by irradiation (Lowry, Bessey & Crawford, 1949; Kalckar, Kjeldgaard & Klenow, 1950).

There exists also the possibility that the apparent activation observed by Bernheim & Dixon (1928) was in reality quite unrelated to the activity of the xanthine oxidase, since any factor which accelerates reduction of the methylene blue or retards its re-oxidation would have the same result. The literature contains a number of reports of light activation of various dehydrogenases as measured by the Thunberg technique. That factors other than a true activation of the enzymes are involved is indicated by the observations that the extent of the accelerated decolorization is dependent upon the particular dye employed (Grönvall, 1937) and that accelerated reduction of MB may occur also with heat-inactivated enzyme preparations and in the absence of substrate (Krestownikoff, 1927). More recently, it has been shown that in the presence of MB, light brings about a dehydrogenation of certain amino-acids and other compounds with concurrent bleaching of the dye (Weil & Maher, 1950; Weil, Gordon & Buchert, 1951). It seems not unlikely that different samples of milk and of crude xanthine oxidase preparations might vary widely in the content of such photolabile substances.

Regardless of the precise mechanism which is involved, it is clear that the apparent activation of xanthine oxidase by light must be of an indirect nature, and hence cannot be adduced as evidence for the direct participation of a flavin enzyme in the indolylacetic acid oxidase system.

SUMMARY

1. Contrary to the results of Bernheim & Dixon (1928), no evidence could be obtained of an activation of xanthine oxidase by light.

2. It is suggested that the apparent activation observed by Bernheim & Dixon was of an indirect nature and may have been due to photodehydrogenation of impurities in their preparation, with concomitant reduction of the methylene blue employed as hydrogen acceptor.

REFERENCES

Bernheim, F. & Dixon, M. (1928). *Biochem. J.* **22**, 113.
Galston, A. W. (1950). *Science*, **111**, 619.
Galston, A. W. & Baker, R. S. (1951). *Amer. J. Bot.* **38**, 190.
Grönvall, H. (1937). *Acta ophthal., Kbh.*, Suppl.
Hodson, A. Z. (1949). *J. Nutrit.* **38**, 25.
Kalckar, H. M., Kjeldgaard, N. O. & Klenow, H. (1950). *Biochim. Biophys. Acta*, **5**, 586.
Kalckar, H. M. & Klenow, H. (1948). *J. biol. Chem.* **173**, 349.
Krestownikoff, A. (1927). *Skand. Arch. Physiol.* **52**, 199.
Lowry, O. H., Bessey, O. A. & Crawford, E. J. (1949). *J. biol. Chem.* **180**, 399.
Parker, C. A. (1949). *Analyst*, **74**, 112.
Taylor, A. H. (1934). *General Electric Review*, p. 410.
Toyama, J. (1933). *Fukuoka-Ikwadaigaku-Zasshi*, **26**, 1483.
Weil, L., Gordon, W. G. & Buchert, A. R. (1951). *Arch. Biochem. Biophys.* **33**, 90.
Weil, L. & Maher, J. (1950). *Arch. Biochem.* **29**, 241.

A Method for the Preparation of Gastrin

By J. E. JORPES, O. JALLING and V. MUTT

Chemistry Department II, Caroline Institute, and the Insulin Laboratory of the Vitrum Company, Stockholm

(*Received* 16 *January* 1952)

Extraction of the hog pyloric mucosa with hot 0·1 N-hydrochloric acid, as applied in the earlier procedures for the preparation of gastrin (Edkins, 1906; Komarov, 1942*a*, *b*; Uvnäs, 1942; Uvnäs, Munch-Petersen & Rönnow, 1944), yields an abundance of inert proteins. In a study of the properties of crude preparations of gastrin we made the observation that the active principle is very soluble in acid methanol. We found this solvent very satisfactory when used for extracting gastrin from hog pyloric mucosa.

Further, we found, during the course of the work, that the main bulk of the activity followed the proteins which flocculated on neutralizing the acid methanolic extract. Since only a few per cent of the total gastrin remained in solution, evaporation of the methanol was superfluous after neutralization. Furthermore, a purification was achieved through fractionating the first methanolic extract at different pH values. The precipitate obtained at pH 5 was as large as that obtained at pH 7, but the latter contained nine-tenths of the gastrin activity. When dissolved in water these fractions flocculated isoelectrically at the same pH values as in methanol. A second small fraction was separated at pH 5·5 in methanol. For the sake of convenience, only the fraction flocculating at pH 7 was used for further purification.

METHODS

Preparation of the hog pyloric mucosa

The stomach was removed not later than 1 hr. after slaughter, emptied of its contents, and everted. The pyloric part, making up a fourth of the stomach, was cut off, rinsed with cold water and immersed for 10 min. in boiling water in order to destroy the enzymes. After cooling, the mucosa was carefully wiped clean of the slimy layer of mucus covering it and dissected free from the underlying muscular tissue. It made up about 20 % of the pyloric part of the stomach. The dissected mucosa was then minced in a meat chopper, frozen and stored for several months at −20°, a procedure which did not affect the yield of gastrin. About forty hogs yielded 1 kg. of mucosa.

Extraction of gastrin

Deeply frozen mucosa (10 kg.) from about 400 animals was minced and suspended in 40 l. 0·1 N-HCl in 95 % (v/v) methanol (solution I: 5 ml. 2 N-HCl + 95 ml. water-free methanol). This and all subsequent operations were carried out at room temperature. After stirring for 2 hr. the suspension was strained through gauze and the cloudy liquor filtered through fluted paper. The clear filtrate was brought to pH 5 with N-NaOH. (All pH measurements were made with the glass electrode.) A heavy precipitate formed at this stage. 5–10 g. of Hyflo Super-Cel were mixed with each litre of the suspension and the mixture filtered with suction. The filter cake contained about one-tenth of the total gastrin activity. The clear filtrate was brought to pH 5·5, when a slight precipitate formed which was filtered off with the aid of Hyflo, and discarded. The filtrate was brought to pH 7 and the precipitate formed allowed to settle for half an hour. The supernatant was then decanted and discarded. The rather voluminous precipitate was either centrifuged down or filtered with the aid of Hyflo Super-Cel. The mother liquor was discarded and the precipitate dissolved in, or extracted with, about 2 l. of 0·1 N-HCl in 99 % (v/v) methanol (solution II: 1 ml. 10 N-HCl + 99 ml. methanol). The dark-brown methanolic solution, which contained about three-fourths of the gastrin activity extracted from the mucosa with methanol can be stored for several weeks at −20° without any loss of activity.

Preparation of purified gastrin

Solution II (6 l., containing extract from about 30 kg. of mucous membrane and 1200 animals) was precipitated by the addition of 24 l. of ethyl ether. The precipitate was removed with suction and the filter cake washed with dry ethyl ether. The ether was allowed to evaporate and the filter cake, usually about 20 g., half of which consisted of inorganic salts, was dissolved in 500–1000 ml. of distilled water. The pH of the solution, about 2·5, was brought to 6·8–7·4 with 0·1 N-NaOH. The precipitate was collected by centrifugation, dissolved in about 200 ml. of 0·05 N-HCl and reprecipitated at pH 7. The precipitate was dissolved in 50 ml. or less of 0·05 N-HCl, 1 l. of methanol was added and the gastrin reprecipitated by the addition of 1 l. of ethyl ether to the clear solution. The precipitate, which contained most of the gastrin, was filtered with suction and dissolved in the smallest possible amount of distilled water (solution *A*).

The addition of 3 l. more of ether to the mother liquor precipitated some more material, which also contained gastrin. The amount was about one-third of that in the first precipitate and the activity per unit wt. was likewise about one-third. Solution *A* was dialysed for 12 hr. at 5° under toluene against distilled water through which carbon dioxide was passed during the dialysis to prevent isoelectric precipitation of the gastrin. The inner fluid was freeze-dried.

The dry powder weighed about 1·5 g. The substance was readily soluble in distilled water and dilute acids. It was fairly stable in the dry state at room temperature, although some loss in activity did occur when it was stored for some months.

The substance is of protein nature and is free of histamine, as evidenced by blood-pressure experiments. When 1 mg. was injected intravenously into a cat, as described by Jalling & Jorpes (1947), it induced in most animals a copious flow of a highly acid gastric juice. The amount secreted by different animals, however, showed great variations. We found values ranging from scarcely any secretion at all in a few animals to 32 ml. 0·1 N-HCl in a single instance. The mean response was about 10 ml. 0·1 N-HCl/mg. gastrin, corresponding to a potency of 10 units/mg., as defined by Jalling & Jorpes. The most potent preparations previously reported are those of Komarov (1942*b*), having an activity of 0·4 unit/mg., and those of Uvnäs (1942), usually with about 0·5 unit/mg. (Kahlson, 1948), and in some instances claimed to vary between 1 and 10 units/mg. (Uvnäs, 1945).

SUMMARY

1. Gastrin has been extracted from boiled hog pyloric mucosa with 0·1 N-hydrochloric acid in 95 % methanol.

2. Impurities were removed on neutralizing the crude extract to pH 5–5·5. The active principle flocculated in the methanolic extract almost quantitatively at pH 7.

3. Further purification was achieved through precipitation from water at pH 7. Inorganic salts were removed through dialysis.

4. 1 mg. given to an anaesthetized cat with vagi cut caused a gastric secretion containing about 10 ml. of 0·1 N-hydrochloric acid, i.e. it contained about 10 secretory units (Jalling & Jorpes, 1947).

This investigation was aided by grants from the Rockefeller Foundation and the Knut and Alice Wallenberg Foundation.

REFERENCES

Edkins, J. S. (1906). *J. Physiol.* **34**, 183.
Jalling, O. & Jorpes, J. E. (1947). *Acta physiol. scand.* **13**, 231.
Kahlson, G. (1948). *Brit. med. J.* **2**, 1091.
Komarov, S. A. (1942*a*). *Rev. canad. Biol.* **1**, 191.
Komarov, S. A. (1942*b*). *Rev. canad. Biol.* **1**, 377.
Uvnäs, B. (1942). *Acta physiol. scand.* **4**, Suppl. 13.
Uvnäs, B. (1945). *Acta physiol. scand.* **9**, 298.
Uvnäs, B., Munch-Petersen, J. & Rönnow, G. (1944). *Acta physiol. scand.* **7**, 389.

The Preparation of Secretin

By J. E. JORPES and V. MUTT

Chemistry Department II, Caroline Institute, and the Insulin Laboratory of the Vitrum Company, Stockholm

(*Received* 16 *January* 1952)

Friedman & Thomas (1950) described a method for the preparation of secretin on a large scale, and in a state of purity permitting its intravenous administration in man. Their method compares favourably with the older ones. Briefly it is as follows: the sodium chloride precipitate of an acid extract of hog intestine is extracted with methanol and the active material precipitated with acetone. Further purification is achieved by means of trichloroacetic acid. The final freeze-dried product has an activity of 100–130 cat units/mg.

We found the method of Friedman & Thomas very useful, although the yield of secretin was fairly low. Friedman & Thomas noted that though vigorous stirring of the intestines during extraction increased the total yield of secretin, so much inert material was extracted at the same time that the secretin activity per unit weight of the final product was lower. We have confirmed this, but prefer to use the more efficient method of extraction, removing the inert protein at a later stage.

The principle introduced by these authors of extracting the secretin from the sodium chloride precipitate with methanol is very valuable. The same cannot, however, be said about the procedure of purification by means of trichloroacetic acid, which precipitates most of the inert proteins together with the active material. In our attempts to avoid using trichloroacetic acid we soon found that the bulk of the inert material was removed on neutralizing the methanolic extract. On adding sodium hydroxide in methanol to pH 7 more than half of the protein matter present in the extract precipitated, while the secretin remained in solution.

The method described below gives, when properly adhered to, a secretin with an activity of from 600 to 900 cat units/mg. ash-free substance, and a yield of 1000–1600 cat units/m. hog intestine.

METHODS

Method of standardization

The method used was that of Wilander & Ågren (1932). Since the technique of the operation has a marked effect on the response of the animals to secretin, we have described our technique in detail.

Cats weighing 2·5–4·0 kg. and starved for 24 hr. were used. 2 hr. before operation the animals were anaesthetized by intramuscular injection of 8·0 ml./kg. body wt. of a warm 1% solution of chloralose in physiological saline.

The abdomen was opened by a 5 cm. longitudinal incision in the mid-line, 2 cm. distal to the sternum. The pylorus was ligated and a 5–6 cm. long loop of intestine, starting from the pylorus, was fixed at each end to the abdominal wall at the ends of the incision.

The intestinal loop was opened by a longitudinal incision 3 cm. in length. The edges of the intestine were folded over the abdominal wall and fastened to it with catgut, at a distance of not less than 6 cm. on each side of the mid-line, The flap of the opened intestine should completely cover the incision in the abdominal wall, and the edges of the latter should not be sewn together since undue pressure on the pancreas must be avoided. The ampulla of Vater should be situated near the middle of the flap of intestine. The great saphenous vein was cannulated.

The cats could be used almost immediately after operation, and were usually in good condition for 12 hr. or more.

The secretin, dissolved in about 1 ml. of physiological saline, was injected into the saphenous vein, followed by 2–3 ml. of warm physiological saline. After a lag of 2–3 min. the secretion of pancreatic juice started abruptly. The juice secreted for the next 10 min. was collected on wads of tissue paper. The wads were soaked in distilled water in an Erlenmeyer flask and brought to boiling with an excess of 0·1 N-HCl. The excess acid was titrated with 0·1 N-NaOH, using methyl red as indicator.

The secretin unit is that amount of secretin which, under the conditions described, induces a secretion of alkali equivalent to 0·1 ml. of 0·1 N-HCl.

Method of preparation

The uppermost 1 m. of the intestine was removed from the hogs not later than 1 hr. after slaughter, emptied of its contents and immersed in ice water. After cooling for 0·5 hr. the intestines were either worked up immediately or frozen and stored at −20°. Storage for several weeks at this temperature did not affect the yield. If frozen material was used it was brought to the laboratory and allowed to thaw in cold water.

The fresh or thawed intestines were everted, the mucus covering them was washed away with ice water and the material squeezed dry. It was then extracted for 0·5 hr. with 200 ml. ice-cold 0·13 N-HCl/m. intestine, with constant stirring during the extraction. When fresh material was used the extraction was performed at the slaughter house.

Volumes of 20 l. extract from 100 m. intestine were brought to the laboratory and left standing overnight in the cold room, care being taken to keep pieces of ice floating in the extract all the time. The supernatant was decanted from the coarse particles that had settled to the bottom, and filtered with suction in the cold room (+4°) after addition of 30 g. of Hyflo Super-Cel/l. The bottom layer, about 1 l., was likewise filtered with the aid of Super-Cel. To each l. of the combined filtrates 320 g. NaCl were added.

The precipitate was collected on a suction filter in the cold room, washed on the filter with cold saturated NaCl solution and pressed as dry as possible. It is essential that the mother liquor is carefully removed before the extraction with methanol. The precipitate weighed about 300–350 g. with 40–60% moisture. It could amount to 500 g. after very thorough shaking during extraction. The precipitate was extracted for 12 hr. at +4° with 5 parts by weight of methanol. Only one extraction was made, as suggested by Friedman & Thomas. The methanol solution which had a pH of about 2·5 (glass electrode) was neutralized to pH 7 with N-NaOH in methanol. A heavy precipitate formed and was removed by filtration through fluted filter paper. It contained only 2–3% of the secretin activity but more than 50% of the original nitrogen extracted. The actual amount of precipitate varied considerably in different preparations due to the extent to which the intestines were stirred during extraction, and to the amount of moisture remaining in the NaCl precipitate. In four instances the amount of nitrogen precipitated when the methanol extract was neutralized made up 49, 40, 60 and 61% respectively of the total amount.

The reaction of the filtrate was adjusted to pH 2·5 with HCl and 3 vol. of cold acetone were added. The precipitate containing the secretin was washed on a suction funnel five times with acetone and three times with absolute ether. Yield, 21–25 g. of air-dry substance. The secretin activity of the dry powder was approximately 10–15 cat units/mg. with a total yield of 2100–3750 cat units/m. of intestine. More than 90% of the precipitate consisted of NaCl, and the N content was 0·5–1·0%.

The acetone precipitate was suspended in 100 ml. ice-cold distilled water per 35 g. of precipitate. This dissolved the NaCl, leaving the secretin undissolved in the concentrated brine which was formed. The suspension was filtered with suction on hardened paper (Schleicher and Schüll no. 1575) and washed with cold concentrated NaCl solution. The filtrate, which contained about 30% of the nitrogen originally present in the acetone precipitate but only a negligible secretin activity, was discarded. The filter cake was dissolved in about 25 ml. cold distilled water. If the solution was cloudy it was cleared by filtration with suction through hardened filter paper after the addition of 1 g. of Hyflo Super-Cel. The clear solution was saturated with NaCl, the precipitate filtered with suction, dissolved in a minute amount of distilled water and freeze-dried. The yield of the dry powder was about 1 g. from 100 m. of intestine. The activity was approximately 200 units/mg. or 2000 units/m.

Freeze-dried powder (10 g. from about 1000 hogs) was suspended in 1000 ml. of cold absolute methanol. Most of the substance dissolved within half an hour; no attention was paid to the undissolved matter. The pH of the suspension was brought to 6·5–7·0 with 0·1 N-NaOH in methanol. The precipitate was removed on a suction filter and dried with ether. It weighed about 6·5 g. and contained about 10% of the total secretin activity of the freeze-dried material.

The pH of the filtrate was adjusted to 2·0–2·5 with 0·1 N-HCl in methanol, and 3 vol. of cold ethyl ether were added. The flocculent white precipitate formed was collected on a suction filter and washed on the funnel with ether. It was immediately dissolved in distilled water and freeze-

dried. Yield, about 2·0 g. with an activity of 400–600 units/mg. or seven to eight times higher than that of the impurities removed at pH 7·0.

The powder was almost water-free, but contained about 35% ash. The secretin activity/mg. organic dry substance is consequently 600–900 units/mg.

SUMMARY

1. Secretin has been extracted with methanol from the sodium chloride precipitate of an intestinal extract, as recommended by Friedman & Thomas.

2. Impurities were removed through repeated isoelectric precipitations from methanol at pH 7.

3. The final product had an activity of from 600 to 900 cat units/mg. ash-free substance, the yield being 1000–1600 units/m. of hog intestine.

This investigation was aided by grants from the Rockefeller Foundation, and the Knut and Alice Wallenberg Foundation.

REFERENCES

Friedman, M. H. F. & Thomas, J. E. (1950). *Proc. Soc. exp. Biol., N.Y.*, **73**, 345.

Wilander, O. & Ågren, G. (1932). *Biochem. Z.* **250**, 489.

The Free Amino-acids of Invertebrate Nerve

By P. R. LEWIS

Physiological Laboratory, University of Cambridge

(*Received* 16 *April* 1952)

Many types of animal tissue possess an electrolyte composition which differs markedly from that of the body fluids in two important respects: first, the inorganic cations, largely sodium and potassium, outweigh the inorganic anions, chloride, sulphate and phosphate, i.e. there is an inorganic anion deficit; and, secondly, the concentration of potassium is greater (and, conversely, the concentrations of sodium and chloride are less) than in the extracellular fluid, i.e. there is an internal accumulation of potassium. This type of electrolyte distribution is highly developed in excitable tissues. Thus, the extruded axoplasm of giant squid nerves contains over 300 m-moles/l. of potassium and only about 50 and 70 m-moles/l. of sodium and chloride respectively, but squid blood contains only about 20 m-moles/l. of potassium and considerably more than 400 m-moles/l. of both sodium and chloride. In both vertebrate and invertebrate muscle tissue various phosphorus compounds account for most of the inorganic anion deficit; and this may be true also for vertebrate nerve which contains a very high concentration of phosphorus, much of which, however, may not contribute to the anion content of the axoplasm since it is combined with nitrogenous bases to form the phospholipins of the myelin sheath. But in the nerves of marine invertebrates such as the Crustacea and the cephalopod molluscs there is much less phosphorus, and here much of the deficit must be made up by purely organic anions.

The function of these internal anions is largely unknown. But they are almost certain to be important in connexion with the maintenance of a high internal potassium concentration, possibly by some mechanism of the type described by Boyle & Conway (1941). It is therefore important to know what the internal anions are, and this paper deals with the identification of the principal ones present in the nerves of certain marine invertebrates.

The first important attempt to identify the anions in these invertebrate nerves was made by Schmitt and his collaborators working first with extruded squid axoplasm (Bear & Schmitt, 1939) and later with aqueous extracts of lobster nerves (Schmitt, Bear & Silber, 1939; Silber, 1941). This work culminated in the isolation by Silber (1941) of large quantities of alanine and aspartic acid, the separate amounts of which he estimated from analyses of the lobster nerve extracts for total amino nitrogen and total amino-acid carboxyl groups assuming that no other amino-acids were present. The values he obtained—133 and 90 m-moles/kg. of alanine and aspartic acid respectively—left a large fraction of the anion deficit unaccounted for. But the presence of other amino-acids would invalidate these values; so a re-investigation of the amino-acid content of these nerves was essential before beginning a search for other anions.

A preliminary, qualitative survey showed that the nerves from all the seven marine invertebrate species tested contained appreciable amounts of

aspartic acid and alanine, and glutamic acid. All contained considerable amounts of a fourth amino-acid; in some this was glycine and in others the aminosulphonic acid taurine. Accurate analyses were therefore made of the free amino-acid content of nerves from three invertebrate species: *Carcinus maenas*, the shore crab, *Sepia officinalis*, the cuttle-fish, and *Homarus vulgaris*, the lobster. For this purpose a micromethod was developed for the estimation of the amino-acids which were first separated by partition chromatography on filter paper. The concentrations of the major inorganic constituents in *Carcinus* nerve were also determined, and a cation-anion balance-sheet drawn up, from which it was clear that, in this tissue at least, very little of the anion deficit remained to be explained. An osmotic balance-sheet was also drawn up and showed that the unexplained osmotic deficit was also small when account was taken of the neutral amino-acids.

METHODS

General techniques

Preparation of material. All the analyses were carried out on freshly dissected tissues. The *Carcinus* leg nerves were dissected out by the procedure described by Keynes & Lewis (1951*a*). The nerve trunks so obtained were 4–5 cm. long; about 5 mm. was cut off each end, and the central portion was briefly, but carefully, blotted on filter paper and weighed on a microbalance. Single samples usually weighed 4–10 mg. Leg nerves from the other Crustacea were prepared in the same way but were normally longer and heavier than those from *Carcinus*. Single *Sepia* axons and samples of squid axoplasm were prepared in the way described by Keynes & Lewis (1951*b*) and averaged 1–2 mg. in weight. The *Carcinus* nerves were dissected out in the Ringer's solution described by Keynes & Lewis (1951*a*); nerves from the other species were dissected out in sea water.

Determination of the inorganic constituents. The values for the Na^+, K^+ and total P contents of *Carcinus* nerve and the ways they were obtained have already been given by Keynes & Lewis (1951*a*, *b*). Cl^- was determined by the colorimetric method of Conway (1935) for amounts between 7 and 35 μg.; pairs of nerves were soaked for at least 4 hr. in 2·5 ml. of water, and two 1 ml. portions then taken for analysis. For the determination of Mg^{++}, the method devised by Robertson & Webb (1939) for the analysis of samples of marine invertebrate blood was adapted to much smaller amounts. This method involves two precipitations with oxine (the second in the presence of only a slight excess of reagent), bromination of the oxine in the precipitate, and titration of the excess BrO_3^- with $Na_2S_2O_4$ after the addition of KI. The procedure used by them was scaled down by a factor of 50 so that analyses on about 30 mg. of nerve were possible. A solution of 0·02 N-$Na_2S_2O_4$ delivered from a horizontal Conway burette was used for the titration, and the filtrations were carried out with a small sintered-glass filter-stick and asbestos pad similar to that used by Cunningham, Kirk & Brooks (1941). An accuracy of 5–10% was obtained; this was sufficient for the present purpose since Mg^{++} is only a minor constituent of these invertebrate nerves.

Estimation of the extracellular space in Carcinus *nerve.* The reference substance used was a levan polysaccharide isolated from Italian rye-grass. A sample of this levan was very kindly supplied by Dr J. Beattie; some of its properties have been discussed by Palmer (1951) and by Bell & Palmer (1949). This polysaccharide was preferred to inulin since, though of a similar mean molecular weight, it is much more soluble in sea water at room temperature and so made possible the analysis of very small samples of tissue. Freshly dissected nerves were soaked in *Carcinus* Ringer containing 1% levan for periods of 2·5–5 hr., and each was then transferred to 1 ml. of distilled water for a similar period. After removal of the nerve residue, the amount of polysaccharide was determined by the method described by Harrison (1942); 2 ml. of the diphenylamine reagent were used for each analysis, and the intensity of the colour produced was determined photoelectrically with the apparatus, described below, which was used for the amino-acid analyses.

Micro-analysis of the amino-acids

The development of a suitable technique. For the determination of the principal free amino-acids in freshly dissected *Carcinus* nerve it would have been possible to use one of the established analytical methods, such as the chromatographic technique of Moore & Stein (1948) or, where applicable, one of the many enzymic methods—sufficient material could have been obtained by pooling many nerves. Some much more sensitive method was needed, however, for the determination of the composition of a single *Sepia* axon and for any investigation of the effect of various factors on the composition of individual *Carcinus* nerves. What was needed was a rapid method of estimating amino-acids in quantities of the order of 0·2–0·5 μg. amino N with an accuracy of about 5%. None of the existing methods satisfied all three requirements of speed, sensitivity and accuracy, but it appeared that colorimetric estimation of the blue compound formed with ninhydrin offered the most promising basis for the development of a suitable technique, the individual amino-acids being first separated by partition chromatography on filter paper.

Development of the colour in aqueous solution after extraction of the amino-acid from the paper did not give sufficiently accurate results, probably because full colour development does not occur in dilute solution. The use of a reducing agent such as $SnCl_2$ is said to improve the reproducibility of colour development (see Landua & Awapara, 1949), but as this also leads to high and variable blank values it is unsatisfactory for the analysis of small samples. Development actually on the paper followed by extraction of the colour was found to give much better results, and the following method of extraction was found to be the most suitable: the circle of paper containing the coloured spot was rolled inside a vertical piece of glass tubing and a jet of steam directed into the open end from above; the colour was rapidly extracted down the paper, and the extract was collected from the lower end of the tube into which was fitted a thin disk of sintered glass to retain any fibres of filter paper detached by the steam. The extraction tube used in the present work was 3 cm. long and tapered from an internal diameter of 8 mm. at the top to one of about 3 mm. at the bottom. In this tube a piece of filter paper 4–5 sq.cm. in area can be extracted completely in 5 min. into a volume of less than 1 ml.

Development of a chromatogram by spraying with a

dilute solution of ninhydrin in butanol and heating to about 80° for 10–20 min. in an oven gives a very variable yield of colour, even the shade of colour varying from day to day. The influence of a number of factors on the colour development was therefore studied, and it was found that the best results could be obtained by adding collidine to the spraying fluid and then developing the chromatogram in a moist atmosphere. The use of collidine was suggested by the observation that chromatograms flowed with this solvent always gave very intense blue spots, and by the work of Harding & MacLean (1915) who found that the rate of formation and the yield of the blue compound produced in aqueous solution with excess ninhydrin were increased in the presence of a weak base such as pyridine. Since this technique was perfected, Woiwod (1949) has advocated the use of collidine in $CHCl_3$ solution. The possibility that the presence of water was necessary for optimum development was suggested by the fact that the formation of the blue compound is essentially an ionic reaction. No further improvement could be obtained by the addition of buffer solutions to the spraying mixture: both adverse pH and high concentrations of salts impair development. Under the conditions used for the quantitative analyses it was found that development was independent of the concentration of ninhydrin in the spraying fluid if this was greater than 0·2% (w/v).

Preparation of nerve extracts. Three methods were used. In the first experiments the nerve was extracted actually on the filter paper with alternate drops of $CHCl_3$ saturated with water and water saturated with $CHCl_3$. This method was used because tracer experiments carried out in this laboratory (Keynes, unpublished work) had shown that saturation of sea water with $CHCl_3$ causes a rapid escape of the internal K^+ from these nerves. Alternatively, the nerve was suspended from a loop of Pt wire and extracted on to the paper, this procedure obviating the removal of the extracted residue from the paper. Extraction of the residues indicated that quantitative transfer of the amino-acids on to the paper was achieved. For the majority of the analyses, however, the nerve was transferred to a tiny test tube containing a weighed quantity of distilled water, and after about 1 hr. portions of the supernatant were transferred to the paper with a micropipette. Control experiments showed that the amino-acids escaped from the nerve with a half-time of 4–6 min., and that after less than 1 hr. the concentrations of amino-acids in the residue of the nerve and in the supernatant were effectively the same. No significant differences were observed in results obtained by the three methods, but the third was adopted for the routine analyses because there were fewer possible sources of error. The *Sepia* axons were soaked in water for only 30 min. since the amino-acids diffuse out very rapidly from these single fibres.

Chromatographic technique. One-dimensional chromatograms were used for the accurate quantitative analyses; samples of nerve extract and known volumes of a standard solution were placed alternately along the starting line at about 3 cm. intervals; and when the third method of extraction was used the same pipette was employed for both the extract and the standard solution. The method of flowing the chromatograms was essentially that used by Dent (1948); phenol and 'collidine' were the two solvents used, phenol always being the first solvent for two-dimensional chromatograms, but ammonia and diethylamine were not added as suggested, since they might interfere with the quantitative determinations. When aspartic and glutamic acids, taurine and alanine were the four main amino-acids present, as was the case in most of the nerve extracts analysed, a complete separation could be obtained with a flow of about 20 cm. in phenol, which took about 7 hr. on the Whatman no. 4 filter paper used. Unfortunately, glycine and taurine coincide on a phenol chromatogram; so when both were present a parallel chromatogram was run in collidine, which separates taurine from all the other amino-acids. The amount of taurine present in the extract was determined from this collidine chromatogram, and the amount of glycine determined by difference from the phenol chromatogram. When the flow was complete, the excess solvent was removed at a temperature below 70° since higher temperatures cause significant losses of amino-acids (see Fowden, 1951).

Development and estimation of the colour formed with ninhydrin. When the paper was dry it was uniformly sprayed with a solution of 0·4% (w/v) ninhydrin in 10% (v/v) aqueous *iso*propanol to which 5% of collidine was added immediately beforehand. In the absence of the collidine this ninhydrin solution is stable indefinitely if protected from sunlight; *iso*propanol was preferred to the more normally used *n*-butanol which has an objectionable smell. The chromatogram was then suspended in a lead-lined tank in the bottom of which was fitted an electrically-heated water bath and a battery of electric light bulbs. Here, in a moist atmosphere at a temperature of 80–90°, development was allowed to proceed for 20–25 min.

Each spot was cut out in turn and extracted, by the method already described, into a tiny test tube containing a few crystals of sodium acetate. The extract was transferred quantitatively to a weighed colorimeter cell and the depth of colour and the weight of solution measured. The colorimetric measurements were made on a Hilger model H 760 Spekker absorptiometer with Ilford 606 filters; special cell-carriages were made so that small 1 cm. cells requiring only about 2·3 ml. of solution could be used. If protected from sunlight the chromatograms did not deteriorate for several hours at least; and the extracts were quite stable when the sodium acetate was included, the colour fading by less than 2% overnight.

Accuracy. In these analyses the composition of the standard solution was so adjusted that the amount of each amino-acid in the standard spot on the chromatogram was within ±25% of that in the sample spot. The amounts of the individual amino-acids in each spot ranged from about 0·025 μmole for glutamic acid and alanine to about 0·10 μmole for aspartic acid. In most of the experiments the standard error of a single estimation was ±4–6%, but occasional chromatograms gave a larger standard error for no predictable reason. Duplicate estimations were normally made, two spots of the extract being included on the same chromatogram.

RESULTS

A preliminary survey

Preliminary, semi-quantitative analyses were made of the principal free amino-acids in nerves from seven species of marine invertebrates—five Crustacea and two cephalopod molluscs—and the results are shown in Table 1. Both one-dimensional and two-dimensional chromatograms of the nerve extracts were run, and the intensities of the spots

compared visually with those of standards flowed in parallel; the spots were not extracted for colorimetric estimation since the main purpose was to make a rapid survey of the occurrence of the individual amino-acids as a preliminary to a more detailed study of one or two individual species. A comparative survey was not contemplated, though such a survey should prove of interest since the variations, particularly of taurine and glycine, observed between different species were far greater than those between individual members of the same species.

Positive identification of the amino-acids

The identities of the five principal amino-acids were confirmed in a number of ways. Identification based solely on the position and colour of spots on chromatograms is inadequate and can be misleading. Thus, when a two-dimensional chromatogram of lobster nerve was run for the first time an intense brown spot with approximate R_F values of 0·41 and 0·22 was observed, and was assumed to be due to asparagine and not to glycine, which flows to almost the same position but is supposed to give

Table 1. *The approximate distribution of the principal free amino-acids in the nerves of some marine invertebrates and in* Carcinus *muscle*

(The results of a preliminary, semi-quantitative survey.)

Species	Tissue	Aspartic acid	Glutamic acid	Alanine	Taurine	Glycine
Carcinus maenas	Nerve	+ + +	+ +	+ +	+ +	−
Cancer pagurus	Nerve	+ + +	+ +	+ +	+ +	±
Maia squinado	Nerve	+ + +	+	+	+	+ +
Homarus vulgaris	Nerve	+ + +	+	+ +	±	+ +
Palinurus vulgaris	Nerve	+ + +	+	+ +	±	+
Sepia officinalis	Nerve	+ + +	+ +	+	+ + +	−
Loligo forbesi	Nerve	+ + +	+ +	+ +	+ + +	±
Carcinus	Muscle	−	±?	+ +	+ + +	+ + +

Probable concentrations in m-moles/kg. wet weight of tissue are: + + + = > 75; + + = 30–90; + = 15–35; ± = 5–15; − = < 5. These ranges are approximate.

Ninhydrin-reacting substances other than the five given in Table 1 were often observed, though in traces only. Thus most of the chromatograms showed a yellow spot a short distance behind the phenol front, which was almost certainly due to proline, and a reddish spot a little further behind, which may have been due to carnosine. Frequently there were one or two faint spots which had moved only a short distance in collidine; these did not correspond to known amino-acids, but they were a normal blue in colour and were probably due to small molecular weight peptides or to simple derivatives of known amino-acids. Up to the present time, however, investigations have been confined to the five amino-acids listed in Table 1, which were responsible for the intense spots.

Since nerve and muscle have many features in common a few samples of invertebrate muscle were also analysed and the results are included for comparison in Table 1. Unlike nerve, *Carcinus* muscle contains no aspartic acid and little if any glutamic; traces of a ninhydrin-reacting substance which could be glutamic acid were found but were insufficient for identification. Alanine, taurine and glycine, however, are all present in large amounts together with a substance, at present unidentified, which flows slightly faster than alanine in phenol but only very slowly in collidine.

the normal blue or purple colour (Dent, 1948). Only later was this spot shown to be due entirely to glycine, which under the development conditions used here sometimes gives a spot that is brown at first and may not turn blue for several hours. The course and speed of colour development with ninhydrin are often of diagnostic value. Aspartic acid goes through a complex series of colour changes; a vivid emerald green can be obtained at an intermediate stage by developing the chromatogram under very dry conditions, and the development of the 'aspartic-acid spot' on nerve chromatograms matches at every stage that of a standard spot flowed in parallel. The colour changes of glycine mentioned above are also typical; and during the development of chromatograms of nerve extracts the spot due to glutamic acid is the first to appear and that due to taurine is the last.

Duplicate two-dimensional chromatograms of *Carcinus* nerve, to one of which appropriate amounts of the four amino-acids had first been added, showed no obvious differences in the distribution of colour in parallel spots; and preliminary treatment with basic copper acetate, as described by Crumpler & Dent (1949), left only the taurine spot unaffected. The separate components were also isolated by the method of Consden, Gordon & Martin (1947) from a 'multi-spot chromatogram' of a *Carcinus* nerve

extract flowed with phenol. The aspartate, glutamate and taurine fractions were found to be stable to the action of 6N-HCl for 24 hr. at 105°; and the glycine fraction similarly isolated from *Carcinus* muscle was also stable to this treatment. (No trace of aspartic acid was formed from the glycine fraction; so asparagine was absent.) The alanine fraction isolated from the nerve extract, however, contained traces of substances other than α-alanine, though this was certainly the major component. Several extra spots appeared on hydrolysis, including one probably due to glutamic acid; so there was definitely a small amount of a peptide present and perhaps also some glutamine. The amounts were small however; and the identity of the major component, which was stable to hydrolysis, cannot be in doubt, particularly in view of the findings of Silber (1941). In the next section the analytical figures given for alanine have not been corrected for the presence of these impurities, but the error thereby introduced should be small and should not materially affect any of the subsequent arguments.

The accurate determination of the amino-acids

The results of accurate analyses of nerves from three species are given in Table 2. Nerves from *Carcinus* and *Sepia* were analysed because they have been used for recent quantitative work in this laboratory and their electrolyte composition was therefore of especial interest. (Squid axons have also been used, but analyses of their axoplasm have not yet been made since the animals would not survive transport to Cambridge.) A few *Homarus* nerves were also analysed because Schmitt and his collaborators used the American lobster for their work, and also partly because *Homarus* was one of the species found to contain considerably more glycine than taurine. The standard errors of the means for *Carcinus* are included in Table 2; since biological variation is the main cause of uncertainty in these results the mean values given for *Sepia* and *Homarus* are probably somewhat less reliable because fewer animals were used for the analyses. Though these results for *Homarus* differ widely from those arrived at by Silber (1941) they would give closely similar values for the total amino N and α-amino CO_2 determinations, upon which Silber's conclusions were based, as can be seen from Table 3 where the two sets of results are compared.

Table 2. *The concentrations of the principal free amino-acids in the nerves of some marine invertebrates*

(All values in m-moles/kg. wet weight of nerve, ±S.E. All ten *Sepia* axons were obtained from three animals.)

Amino-acid	*Carcinus* whole leg nerve	*Sepia* single axon	*Homarus* whole leg nerve
Aspartic acid	138±4·5	82	112
Glutamic acid	35±1·5	39	25
Alanine	33±3·5	21	33
Taurine	65±5	103	*c* 12
Glycine	<5	<5	35
Anion total	173±5	122	137
No. of analyses	16	10	8
No. of nerves analysed	9	10	3

Table 3. *The amino-acids of lobster nerve; a comparison of the amino* N *and α-amino* CO_2 *values obtained by Silber* (1941) *with those deduced from results given in Table* 2

(All values are expressed as m-moles/kg. of nerve. The experimental procedure used by Silber was stated (Silber & Schmitt, 1940) to reveal the second carboxyl group of aspartate to the extent of 90% and that of glutamate to the extent of 3%; and the values for the α-amino CO_2 were calculated on the basis of these figures.)

	Concentration of amino-acid	Calculated values for: Amino N	Calculated values for: α-Amino CO_2
From values deduced by Silber:			
Aspartate	90	90	171
Alanine	133	133	133
Totals		223	304
From values given in Table 2:			
Aspartate	112	112	213
Glutamate	25	25	26
Alanine	33	33	33
Taurine	12	12	0
Glycine	35	35	35
Totals		217	307

The cation-anion balance in Carcinus *nerve*

In order to assess the importance of these dicarboxylic amino-acids as internal anions a cation-anion balance-sheet was drawn up for *Carcinus* nerve and is reproduced in Table 4. The concentrations of the major constituents (sodium, potassium, magnesium, chloride, total phosphorus, aspartate and glutamate) were determined by direct analysis of freshly dissected nerves. There is considerable biological variation; so sufficient analyses were made to reduce the standard error of the mean for each constituent to about 4 m-equiv./kg. or less. The concentrations of sodium, potassium and total phosphorus were of interest in other connexions, and values have already been published by Keynes & Lewis (1951*a*, *b*). Values of 257 and 262 m-moles/kg. for the potassium content were obtained by two different methods of analysis and 260 is used here as a reasonable average figure. Values for some minor constituents were arrived at from figures given in the literature for other marine crustacean nerves; variations between species are unlikely to introduce a large absolute error since the estimated total contribution made by the ions concerned (calcium, bicarbonate, lactate and keto acids) is only about 50 m-equiv./kg., i.e. some 6% of the total electrolyte

content. Estimation of the total anion contribution made by the phosphorus compounds was complicated by the fact that no one complete analysis of them appears to have been made. Use was therefore made of two partial analyses of the phosphorus fractions in *Homarus* nerve, those of the acid-soluble fraction by Engel & Gerard (1935) and of the lipid fraction by Brante (1949), from which the total anion contribution in *Carcinus* was estimated on the assumption that the relative distribution of phosphorus compounds is the same in both species. It was also assumed that the pH of the axoplasm was about 7·0. Such an estimate is obviously only approximate but is unlikely to be in error by much more than 5 m-equiv./kg.

Table 4. *The cation-anion balance in* Carcinus *nerve*

(All values are expressed as m-equiv./kg. wet weight.)

	Ionic concentrations		
Electrolyte	Mean ± S.E.	Group totals	Anion deficit remaining
Sodium	152 ± 5	—	—
Potassium	260 ± 4	—	—
Magnesium	23 ± 3	—	—
Calcium*	13	448	448
Chloride	145 ± 2	—	—
Sulphate†	9	—	—
Bicarbonate‡	9	163	285
Phosphorus compounds§	45	—	—
Keto acids‡	14	—	—
Lactate‖	13	72	213
Aspartate	138		
Glutamate	35	± 5 173	40

* Value obtained by Tipton (1934) for *Libinius* and *Homarus*.

† Calculated value for sulphate in the extracellular space where it is assumed to be at the same concentration as in Ringer.

‡ Values calculated from figures of Silber's (1941) for *Homarus*.

§ The method used to estimate the contribution of the phosphorus compounds is given in the text.

‖ Value obtained by Holmes (1929) for *Cancer*.

No account has been taken of the base-binding power of the proteins of the axoplasm or of any peptides that may be present. The dicarboxylic amino-acids are assumed to bind one equivalent of cation and the mono-carboxylic amino-acids none; the small contribution which taurine may make by virtue of its strongly acidic sulphonic acid group is neglected. Values are given in the table for lactate and keto acids; but there are a number of other acidic intermediates of carbohydrate metabolism, such as acetic and succinic acids, which may be present in significant amounts. The presence of organic cations would naturally increase the anion deficit, but they rarely occur in high concentrations in biological tissues. All these omissions from the table do not, however, radically affect the general picture or the conclusion that the anion deficit which remains unexplained is quite small. That the deficit due to unidentified small molecular weight anions may be even less than indicated is suggested by a few analyses of the nerve residues obtained after soaking in distilled water to release the amino-acids. When calculated in terms of m-moles/kg. of water, the concentrations of taurine and alanine in the nerve residue approximated to those in the supernatant and the concentrations of aspartic and glutamic acids were a little less, though not significantly so, whereas some 8 % of the total sodium and potassium of the nerve and 45 % of the total phosphorus were retained in excess of that necessary for equivalence with the supernatant. These results suggest that the nerve residue contains fixed, or very slowly diffusing, anions which set up a Donnan equilibrium between the interior of the residue and the supernatant; and this would account for the fact that Silber (1941) failed to extract the last 10 % of the cations from lobster nerve. It is obvious that the unextracted phosphorus must account for much of the cation-binding power of the residue; but if the value of 45 m-equiv./kg. given in Table 4 for the total anion contribution of phosphorus compounds is correct, about 15 m-equiv./kg. of sodium and potassium must be retained by other anions. These considerations reduce the overall deficit to the order of 25 m-equiv./kg., a value so small that there is unlikely to be any organic anion in *Carcinus* nerve present in amounts comparable with those of aspartate and glutamate. This conclusion is not justified for *Sepia*, however, where the balance does not appear to be so complete since the sum of aspartate and glutamate is about 50 m-equiv./kg. less than in *Carcinus*, although the sum of sodium and potassium is almost the same. Owing to the very small weight of a single *Sepia* axon no attempt has yet been made to look for other anions which might account for this difference. *Homarus* appears to be intermediate, and although aspartate is certainly the principal organic anion there may be unidentified anions which are as important as glutamate. Nevertheless, it is true to say that in all the seven invertebrate species studied aspartate and glutamate must make up a large fraction of the total internal anions and therefore balance much of the potassium present.

The osmotic balance in Carcinus *nerve*

Just as the aspartate and glutamate help to make up the total anionic concentration necessary to balance the internal potassium, so, it may be argued, the other amino-acids help to make up the total solute concentration necessary to maintain osmotic equilibrium with the blood. This appears to be a justifiable argument since Hill (1950) has concluded that single *Sepia* axons behave as almost perfect osmometers so that the maintenance of the

internal osmotic pressure is probably important for optimum functioning of the nerves. An approximate osmotic balance sheet was therefore drawn up for *Carcinus* nerve and is reproduced in Table 5. Until more is known about the physical conditions existing in the axoplasm an exact treatment of this problem is impossible; so in the table all the solute species are assumed to have osmotic activities proportional to their molal concentrations. Only

Table 5. *The osmotic balance in* Carcinus *nerve*

	Nerve		Ringer
Water content ...	865 g./kg.		992 g./l.
Osmotic activities ...	(mg. ions/kg. nerve)		(mg. ions/l. Ringer)
	Individual activities	Running total	
Sodium	152	152	495
Potassium	260	412	11
Chloride	145	557	532
Calcium, magnesium and sulphate	12	569	49
		Total	1087
Acid-soluble phosphorus compounds	16	585	
Bicarbonate, lactate and keto acids	36	621	—
Aspartate and glutamate	173	794	—
Taurine and alanine	98	892	—
Unexplained deficit	56	948	—
Totals (in mg. ions/kg. water)		1096	1096

that part of the calcium and magnesium which is in the extracellular space is included since most of the intracellular fraction is probably bound to proteins. The osmotic contribution of the proteins themselves is also neglected but is certainly quite small. The unexplained osmotic deficit is small (56 mg. ions/kg. of nerve) and is little larger than the unexplained anion deficit; so it is unlikely that appreciable amounts of undiscovered solutes exist within the axoplasm. The osmotic deficit would be considerable but for the taurine and alanine which together form more than 10 % of the total solutes in these nerves.

The extracellular space of Carcinus *nerve and the probable intracellular ionic concentrations*

It is often desirable to know the actual electrolyte concentrations in the axoplasm, and since these can be calculated from whole-nerve analyses only if the fraction of extracellular space is known, an attempt was made to measure this fraction in *Carcinus* nerve. The mean of seven determinations was $0{\cdot}242 \pm 0{\cdot}011$ l./kg. This value is probably slightly low since it was obtained with a polysaccharide sample which, though it had a mean molecular weight of 5000–6000, almost certainly contained traces of high molecular weight material which might penetrate some parts of the true extracellular space only very slowly. A maximum value of 0·272 l./kg. can be deduced, however, from the figures for the chloride concentrations, 532 m-moles/l. in the *Carcinus* Ringer and 145 m-moles/kg. in the fresh nerve, on the assumption that the nerve axoplasm contains no chloride, but this value is probably too high since the large fibres in both squid and *Sepia* have been shown to contain appreciable internal chloride (Steinbach, 1941; Keynes & Lewis, 1951*b*). Thus the true mean value for the extracellular space can reasonably be assumed to lie between 0·24 and 0·27, and these two extremes were used to calculate the pairs of values for the intracellular concentrations given in Table 6.

DISCUSSION

This investigation began as a search for the organic anions which balance the internal potassium of invertebrate nerves. It has shown that the amino-acids are a conspicuous group of electrolytes in these nerves; in *Carcinus* nerve, for example, the aspartate and glutamate are together sufficient to balance about two-thirds of the internal potassium, and the alanine and taurine, whilst they have no significant cation-binding power, contribute over 10 % of the total osmotic pressure of the axoplasm.

These amino-acids undoubtedly exist in the free state in the axoplasm. When a nerve trunk is placed in distilled water they come out with a half period of about 5 min. compared with a half period of about 3 min. for the loss of radioactive sodium ions from the extracellular spaces of a nerve placed in inactive Ringer (Keynes & Lewis, 1951*a*). Thus the half period for the escape of the amino-acids is so short that their release from the axoplasm must be very rapid, so rapid that their formation by enzymic action would be impossible since the 'free'

Table 6. *The probable intracellular concentrations of the principal electrolytes in* Carcinus *nerve*

	Concentration		Quantity in 1 kg. of nerve		Probable intracellular concentration
Electrolyte	In nerve (m-moles/kg.)	In Ringer (m-moles/l.)	Extracellular (m-moles)	Intracellular (m-moles)	(m-moles/kg. water)
Sodium	152	495	119–134	33–18	53–30
Potassium	260	11	3	257	412–432
Chloride	145	532	128–144	17–1	<27
Aspartate and glutamate	173	—	—	173	276–291
Alanine and taurine	98	—	—	98	157–165

amino-acids form 25 % of the total dry weight of the nerve!

Substances present in such large amounts are likely to have important functions to perform. It seems reasonable to suppose that two very important functions of these amino-acids are to provide, on the one hand, the anions needed to balance the essential internal cations, and, on the other hand, the extra solutes necessary for osmotic equilibrium; and support for this supposition is provided by the two balance sheets drawn up for *Carcinus* nerve since both the cation-anion and the osmotic balances are very close when account is taken of the contributions made by the amino-acids.

The distribution of free amino-acids in other excitable tissues differs markedly from that found in these invertebrate nerves. Thus *Carcinus* muscle (see Table 1) contains little or no aspartate or glutamate but large quantities of alanine, taurine and glycine, and Camien, Sarlet, Duchateau & Florkin (1951) have recently reported the presence of large amounts of glycine and proline in *Homarus* muscle. Taurine has long been known to occur in marine invertebrates. Kelly (1904) isolated it from *Mytilus* muscle, which from her results would appear to contain about 120 m-moles/kg., and similar amounts have been reported to occur in other marine creatures. (Kelly (1904) appears to have been misquoted by several authors. Her value of 5% for the taurine content of *Mytilus* muscle undoubtedly refers to tissue which had been dried at 110°, but Webb & Young (1940) and Krogh (1939) assume that it refers to fresh tissue: since the dry weight is about one-third of the wet weight, fresh *Mytilus* muscle must, in fact, contain only about 1·6 % taurine.) Baldwin (1947) has suggested that it may play an important part in regulating the intracellular osmotic pressure, and this suggestion is probably correct not only for taurine, but also for other non-toxic, nitrogenous substances, such as glycine, glycine betaine and trimethylamine oxide, which have been reported to occur in significant amounts in the muscle tissues of various marine invertebrates. The comparative absence of aspartate and glutamate in invertebrate muscle may be correlated with the fact that a large fraction of the internal potassium can be balanced by organic phosphorus compounds which are presumably associated with the contractile mechanism, for Engel & Gerard (1935) found a total of 66 m-moles/kg. acid-soluble phosphorus in *Limulus* muscle compared with only 13 m-moles/kg. in *Homarus* nerve.

Vertebrate excitable tissues, on the other hand, contain only small amounts of free amino-acids. Awapara, Landua & Fuerst (1950) have made a fairly comprehensive survey of the amino-acid content of the tissues of the rat; their values for the excitable tissues have been recalculated and are given in Table 7 in m-moles/kg. of fresh tissue. The relative distribution of the individual amino-acids is closely similar to that found in the invertebrates:

Table 7. *Distribution of free amino-acids in the excitable tissues of the rat*

(Data of Awapara *et al.* (1950) recalculated: all values in m-moles/kg. wet weight of tissue.)

Amino-acid	Muscle	Brain
Aspartate	<1	3·6
Glutamate	1·7	9·2
Alanine	4·4	<1
Taurine	11·6	2·4
Glycine	5·7	<1

taurine and alanine predominate in muscle and the dicarboxylic amino-acids predominate in nerve (i.e. brain), though the proportion of aspartate in the latter is small. Similar values have been reported in other mammalian excitable tissues, and it has been confirmed that concentrations of the same order exist in several types of vertebrate brain and peripheral nerve.

Thus, of the tissues discussed above, only invertebrate nerve contains more than a few m-moles/kg. of aspartate, and its presence there in such large amounts is most probably due to the need for maintaining a very high internal potassium concentration. Similarly, the fact that invertebrate excitable tissues contain far higher concentrations of the three neutral amino-acids than do the vertebrate ones is probably a direct consequence of the much higher internal osmotic pressure which the marine invertebrates have to maintain. Glutamate, though its concentration is less in vertebrate than in invertebrate nerve, balances about the same fraction of the potassium in both; so it may, perhaps, have a fundamental part to play in nervous conduction. These amino-acids may, of course, have important biochemical roles to play since alanine, aspartate and glutamate are all closely connected with aerobic glycolysis; and it is perhaps significant that glutamate appears to be one of the key substances in the biochemistry of brain (see, for example, Weil-Malherbe, 1950; Terner, Eggleston & Krebs, 1950). But in these invertebrate nerves it seems likely that aspartate and glutamate are important principally as internal anions, possibly as indiffusible anions which thereby set up some form of a Donnan equilibrium similar to that suggested by Boyle & Conway (1941) for frog muscle.

SUMMARY

1. A study has been made of the amino-acids present in aqueous extracts of nerves from certain marine Crustacea and cephalopod molluscs.

2. Nerves from all seven species tested contained large amounts of aspartic acid, glutamic acid and alanine, plus taurine in some species and glycine in others. These amino-acids account for over 20% of the dry weight of the nerves and are almost certainly in free solution in the axoplasm.

3. The concentrations of these amino-acids present in three species were accurately determined by a micromethod developed for the purpose: paper chromatograms of the nerve extracts were developed with ninhydrin and the amount of colour extracted with steam was estimated photoelectrically. As little as 0·05 μmole of an individual amino-acid could be estimated with a standard error of about $\pm 5\%$.

4. The major inorganic electrolytes in *Carcinus maenas* nerve were estimated, and balance-sheets were drawn up for the cation-anion and osmotic balances, in both of which the unexplained deficit was small when account was taken of the free amino-acids present.

5. The possible functional significance of these amino-acids is briefly discussed: it is suggested that the dicarboxylic amino-acids function as internal anions to balance the high internal potassium concentration necessary for nervous conduction and that the other, 'neutral', amino-acids function as solute species to maintain the high internal osmotic pressure necessary for equilibrium with sea water.

I wish to thank Dr R. D. Keynes for his help and advice, and Prof. A. L. Hodgkin, F.R.S., for his continued interest in this work. I am also indebted to the Marine Biological Association for constant assistance in obtaining experimental animals. This work was made possible by a grant from the Rockefeller Foundation, to whom I wish to express my gratitude.

REFERENCES

Awapara, J., Landua, A. J. & Fuerst, R. (1950). *Biochim. Biophys. Acta*, **5**, 457.
Baldwin, E. (1947). *Dynamic Aspects of Biochemistry*. Cambridge University Press.
Bear, R. S. & Schmitt, F. O. (1939). *J. cell. comp. Physiol.* **14**, 205.
Bell, D. J. & Palmer, A. (1949). *Biochem. J.* **45**, xiv.
Boyle, P. J. & Conway, E. J. (1941). *J. Physiol.* **100**, 1.
Brante, G. (1949). *Acta physiol. scand.* **18**, Supplement 63.
Camien, M. N., Sarlet, H., Duchateau, G. & Florkin, M. (1951). *J. biol. Chem.* **193**, 881.
Consden, R., Gordon, A. H. & Martin, A. J. P. (1947). *Biochem. J.* **41**, 590.
Conway, E. J. (1935). *Biochem. J.* **29**, 2221.
Crumpler, H. R. & Dent, C. E. (1949). *Nature, Lond.*, **164**, 441.
Cunningham, B., Kirk, P. L. & Brooks, S. C. (1941). *J. biol. Chem.* **139**, 21.
Dent, C. E. (1948). *Biochem. J.* **43**, 169.
Engel, G. L. & Gerard, R. W. (1935). *J. biol. Chem.* **112**, 379.
Fowden, L. (1951). *Biochem. J.* **48**, 327.
Harding, V. J. & MacLean, R. M. (1915). *J. biol. Chem.* **20**, 217.
Harrison, H. E. (1942). *Proc. Soc. exp. Biol., N.Y.*, **49**, 111.
Hill, D. K. (1950). *J. Physiol.* **111**, 304.
Holmes, E. G. (1929). *Biochem. J.* **23**, 1182.
Kelly, A. (1904). *Beitr. chem. Physiol. Path.* **5**, 377.
Keynes, R. D. & Lewis, P. R. (1951*a*). *J. Physiol.* **113**, 73.
Keynes, R. D. & Lewis, P. R. (1951*b*). *J. Physiol.* **114**, 151.
Krogh, A. (1939). *Osmotic Regulation in Aquatic Animals*. Cambridge University Press.
Landua, A. J. & Awapara, J. (1949). *Science*, **109**, 385.
Moore, S. & Stein, W. H. (1948). *J. biol. Chem.* **176**, 367.
Palmer, A. (1951). *Biochem. J.* **48**, 389.
Robertson, J. D. & Webb, D. A. (1939). *J. exp. Biol.* **16**, 155.
Schmitt, F. O., Bear, R. S. & Silber, R. H. (1939). *J. cell. comp. Physiol.* **14**, 351.
Silber, R. H. (1941). *J. cell. comp. Physiol.* **18**, 21.
Silber, R. H. & Schmitt, F. O. (1940). *J. cell. comp. Physiol.* **16**, 247.
Steinbach, H. B. (1941). *J. cell. comp. Physiol.* **17**, 57.
Terner, C., Eggleston, L. V. & Krebs, H. A. (1950). *Biochem. J.* **47**, 139.
Tipton, S. R. (1934). *Amer. J. Physiol.* **109**, 457.
Webb, D. A. & Young, J. Z. (1940). *J. Physiol.* **98**, 299.
Weil-Malherbe, H. (1950). *Physiol. Rev.* **30**, 549.
Woiwod, A. J. (1949). *J. gen. Microbiol.* **3**, 312.

A Method for the Quantitative Determination in Urinary Extracts of C_{21} 17:20-Dihydroxy-20-methylsteroids

By R. I. COX

Department of Biochemistry, University of Edinburgh

(*Received* 7 *March* 1952)

Hyperactivity of the adrenal cortex in human subjects due to malignant neoplasm, hyperplasia or stimulation by adrenocorticotropic hormone (ACTH) has frequently been observed to be associated with the urinary excretion of considerable amounts of various C_{21} 20-methylsteroids (see Table 1).

Table 1. *20-Methylsteroids, probably of adrenal origin, isolated from human urine*

Compound	Associated condition of subjects	References
Pregnane-3α:17α:20α-triol	Adrenal hyperplasia	Butler & Marrian (1937, 1938)
	Adrenal hyperplasia	Mason & Kepler (1945)
	Adrenocortical tumour	
Pregnane-3α:17α-diol-20-one	Adrenal hyperplasia and adrenocortical tumour	Lieberman & Dobriner (1945)
	Adrenal hyperplasia	Miller & Dorfman (1950)
Pregn-5-ene-3β:17α:20α-triol	Adrenocortical tumour	Hirschmann & Hirschmann (1950)
Pregn-5-ene-3β:17α-diol-20-one	Adrenocortical tumour	Hirschmann & Hirschmann (1947)
Pregnane-3α:20α-diol-11-one	Adrenal hyperplasia	Lieberman, Fukushima & Dobriner (1950)
Pregnan-3α-ol-11:20-dione	Adrenal hyperplasia	Lieberman *et al.* (1950)
Pregnane-3α:17α-diol-11:20-dione	After administration of ACTH and cortisone to patients with neoplasia	Lieberman *et al.* (1951)

Such urinary 20-methylsteroids may arise directly from related 20-methylsteroids present in the adrenal glands, but there is good reason to suppose that they may in part arise by metabolic reduction at C-21 of 21-hydroxysteroids secreted by the glands. The evidence that such metabolic reduction of 21-hydroxysteroids to 20-methylsteroids, i.e. to 21-deoxysteroids can occur in the human body appears to be conclusive. Thus Cuyler, Ashley & Hamblen (1940) and Horwitt, Dorfman, Shipley & Fish (1944) isolated pregnane-3α:20α-diol from the urine of human subjects after the administration of 11-deoxycorticosterone acetate; Mason (1948) isolated pregnane-3α:20α-diol-11-one after the administration of 11-dehydrocorticosterone; while more recently Lieberman, Hariton, Stockem, Studer & Dobriner (1951) have isolated pregnane-3α:17α-diol-11:20-dione after administering cortisone acetate. It has been suggested that the principal C_{21} adrenocortical hormone secreted by the adrenal glands in man may be 17-hydroxycorticosterone (Reich, Nelson & Zaffaroni, 1950; Hechter, 1950; Pincus, Hechter & Zaffaroni, 1951). In view of this possibility and because of the widespread therapeutic use of 17-hydroxy-11-dehydrocorticosterone (cortisone), it was thought that a specific method for the quantitative determination in urine of C_{21} 17:20-dihydroxy-20-methylsteroids which might be formed from these two hormones (and from 17-hydroxy-11-deoxycorticosterone) by metabolic reduction of the C-21 hydroxyl group, would be of some value and interest.

As is well known, C_{21} steroids with either α-ketol or α-glycol side chains give almost quantitative yields of formaldehyde on oxidation with periodic acid, and this reaction has been widely employed for the quantitative determination in suitably prepared urine extracts of the so-called 'formaldehydogenic' steroids (Lowenstein, Corcoran & Page, 1946; Corcoran & Page, 1948; Daughaday, Jaffe & Williams, 1948). On oxidation with periodic acid, C_{21} 17:20-dihydroxy-20-methylsteroids, and steroids of no other type, should yield acetaldehyde. Accordingly, the possibility was envisaged of determining both formaldehydogenic and acetaldehydogenic steroids in urinary extracts by periodate oxidation with separation and estimation of the formaldehyde and acetaldehyde thus produced. Talbot & Eitingon (1944) have estimated the 17-ketosteroids formed after periodate oxidation of urine extracts, thus measuring the combined total of C_{21} 17:20:21-trihydroxysteroids and C_{21} 17:20-dihydroxy-20-methylsteroids.

With pregnane-3α:17α:20α-triol (Butler & Marrian, 1937, 1938) preliminary experiments showed that nearly quantitative yields of acetaldehyde as determined colorimetrically in sulphuric acid with 4-hydroxydiphenyl (Miller & Muntz, 1938; Koenemann, 1940; Barker & Summerson, 1941) could be obtained after oxidation with periodic acid. In further experiments in which mixtures of pregnane-3α:17α:20α-triol and 11-deoxycorticosterone or 17-hydroxy-11-deoxycorticosterone were oxidized with periodic acid, it was found possible to remove the acetaldehyde quantitatively from a reaction mixture containing glycine without loss of formaldehyde, by aeration into a bisulphite trap (cf. Shinn & Nicolet, 1941). The formaldehyde could then be recovered quantitatively in the usual way by distillation (Daughaday *et al.* 1948).

To test out the potentialities of the procedure for the determination of acetaldehydogenic steroids in urine, recovery experiments were carried out in which known amounts of pregnane-3α:17α:20α-triol were added to both unwashed and sodium hydroxide-washed chloroform extracts of urine from normal men. These extracts were obtained from fresh urine, from urine after incubation with ox-spleen β-glucuronidase at pH 4·5, and from urine extracted after standing at room temperature at pH 1 for 24 hr. In every experiment 'blank' determinations were carried out on a portion of the extract with no added pregnanetriol.

Reasonably satisfactory recoveries were obtained in those experiments in which the triol was added to sodium hydroxide-washed chloroform extracts of urine. In those in which unwashed extracts were employed, the recoveries were irregular, indicating possibly the presence of material interfering in some way with the periodate oxidation of the triol. It may be noted that Hollander, Di Mauro & Pearson (1951), studying the recovery in periodate oxidations of 11-deoxycorticosterone added to urine extracts, concluded that the oxidation could be inhibited by substances present in crude extracts.

The 'blank' experiments all indicated the presence of acetaldehydogenic material in the extracts, and it is noteworthy that from the various extracts the amounts of acetaldehyde obtained were roughly of the same order as those of formaldehyde and, like the latter, were increased both by glucuronidase and acid hydrolysis of the urine. It is also noteworthy that there was little or no increase in chloroform extractable acetaldehydogenic material over that obtained from untreated urine when incubation with glucuronidase was carried out in the presence of saccharate. Since the latter is a competitive inhibitor of β-glucuronidase (Karunairatnam & Levvy, 1949), this supports the suggestion that acetaldehydogenic material released during enzymic incubation was present as a β-glucuronide. It is pertinent to note that Mason & Kepler (1945) isolated from adrenal carcinoma urine a mixture of steroid conjugates which yielded pregnane-3α:17α:20α-triol after incubation with a crude rat-liver glucuronidase preparation.

As far as can be judged from recovery experiments with pregnane-3α:17α:20α-triol, the procedure developed should provide a satisfactory method for determining acetaldehydogenic steroids in sodium hydroxide-washed chloroform extracts of untreated urine, and of urine hydrolysed enzymically or with acid. It is not claimed that this procedure provides a means for the quantitative determination of such steroids in urine, since no demonstrably quantitative methods have yet been developed for the hydrolysis of the conjugated acetaldehydogenic steroids which must be assumed to be present in urine, or for the extraction of the free steroids after hydrolysis. In fact, in these respects the method is neither better nor worse than the widely employed procedures for the determination of urinary reducing or formaldehydogenic steroids, the difficulties involved in which have been recently summarized by Marrian (1951).

EXPERIMENTAL

Apparatus. The apparatus used is shown in Fig. 1. *A* is the oxidation tube; *B* contains aqueous $NaHSO_3$ solution in which acetaldehyde, carried over from the oxidation mixture in the air stream, is trapped. A second bisulphite trap has not been found necessary under the conditions used.

Pregnane-3α:17α:20α-triol, m.p. 245–247° (Butler & Marrian, 1938) has been used throughout as the reference acetaldehydogenic steroid. All steroid samples were dried *in vacuo* over P_2O_5 before weighing. Standard solutions were prepared in redistilled ethanol.

Method. Ethanolic solutions of pure steroids or of suitably prepared urine extracts are evaporated in oxidation tubes *A* (Fig. 1) to about 1 ml. under a rapid stream of filtered air on a water bath at about 80°. Evaporation to dryness is carried out *in vacuo* with warming on a water bath. The residues are moistened with 0·10 ml. glacial acetic acid (distilled off CrO_3). The tubes are warmed slightly in a water bath and rotated to wet any residue adhering to the sides of the tube. Equal volumes of 0·12 M-periodic acid in 0·4 N-H_2SO_4 (A.R.) and 1 % glycine in 0·4 N-H_2SO_4 (A.R.) are freshly mixed, and 1 ml. portions of the mixture pipetted into the oxidation tubes. The trap tubes *B*, each containing 2 ml. 1 % $NaHSO_3$ solution, are immediately connected up. A slow stream of air, purified by passage through conc. H_2SO_4 (A.R.) and a soda-lime tower is blown through the sets of tubes arranged in parallel. The rate of aeration is about 5 ml./min.

After 45 min. of oxidation with aeration, the air flow is stopped and the tubes are disconnected. The formaldehyde retained in the oxidation mixture is estimated as in the method of Daughaday *et al.* (1948), using 3 ml. 6 % (w/v) $SnCl_2$ (A.R.) solution to reduce the correspondingly larger excess of periodic acid present.

The bisulphite traps are made up to 3 ml. by washing down the ends of the connecting tubes with distilled water,

and 1 ml. samples of the solutions pipetted into dry 150 × 25 mm. test tubes. The colorimetric estimation of acetaldehyde in these samples is then carried out according to Barker & Summerson (1941), except that 7·0 ml. portions of conc. H_2SO_4 (A.R.) are added to the samples, and colour development is carried out at 25°.

The absorption of the solutions is determined with a Hilger Spekker absorptiometer using Ilford no. 605 filters.

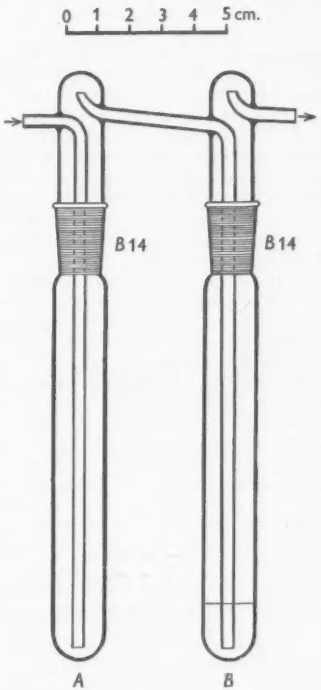


Fig. 1. Apparatus used for oxidation with aeration. *A*, oxidation tube; *B*, bisulphite trap graduated at 3·0 ml. Arrow indicates direction of air stream.

Colorimetric estimation of acetaldehyde

The method of Miller & Muntz (1938), as modified by Koenemann (1940) and Barker & Summerson (1941), must be rigorously standardized to obtain reproducible results. Particular attention should be paid to the purity of the A.R. H_2SO_4. For instance, Russell (1944) has shown that presence of nitrates or nitrites in the sulphuric acid affects the colour development. Therefore each bottle of H_2SO_4 should be checked before use and those giving poor colour development discarded.

Also to be noted is the advice of Barker & Summerson (1941) against the use of chromic acid mixtures for cleaning apparatus used in the acetaldehyde estimations, because of the difficulty of removing interfering traces of the cleaning agent.

Optimum conditions for the oxidation of pregnane-3α:17α:20α-triol

The effects of varying periodic acid concentration, time of aeration and acidity of the oxidation mixture on the yield of acetaldehyde obtained by periodate oxidation of pregnanetriol are illustrated in Tables 2–4. Apart from the variable studied, other conditions in these experiments were as described in the method. The oxidation-aerations have been carried out at room temperature, i.e. 13–21° and within this range the recoveries of acetaldehyde have not been affected by temperature.

Table 2. *Effect of periodic acid concentration on oxidation of pregnane-3α:17α:20α-triol*

(58·0 μg. pregnanetriol samples oxidized.)

Concn. of periodic acid (M)	Acetaldehyde found (μg.)	Equivalent amount of triol (μg.)	Recovery (%)
0·24	7·80	59·5	103
	7·80	59·5	103
0·12	7·61	58·0	100
	7·80	59·5	103
0·04	7·87	60·0	103
	6·69	51·0	88
0·01	5·50	42·0	72
	6·69	51·0	88

Table 3. *Effect of oxidation aeration time on yield of acetaldehyde from pregnane-3α:17α:20α-triol oxidized with* 0·06 M *-periodic acid*

(25·1 μg. pregnanetriol samples oxidized.)

Time aerated (min.)	Acetaldehyde found (μg.)	Equivalent of triol (μg.)	Recovery of triol (%)
5	0·11	0·9	4
10	0·45	3·4	13
15	0·62	4·7	19
20	1·13	8·6	34
25	2·29	17·4	69
30	3·07	23·4	93
40	3·32	25·2	100
50	3·27	24·9	99

Table 4. *Effect of acidity of oxidation mixture on yield of acetaldehyde from pregnane-3α:17α:20α-triol in periodate oxidation*

(25·1 μg. pregnanetriol samples oxidized.)

Normality of H_2SO_4 in oxidation mixture	Acetaldehyde found (μg.)	Equivalent amount of triol (μg.)	Recovery of triol (%)
0	0·87	6·7	27
0·01	2·31	17·6	70
0·05	2·97	22·8	90
0·1	2·76	21·1	84
0·2	3·21	24·5	98
0·4	3·35	25·6	102
0·8	3·23	24·7	98

Table 5 shows the recoveries of acetaldehyde after oxidation of pregnanetriol with 0·06 M-periodic acid in 0·4 N-H_2SO_4 with aeration for 45 min. as in the method finally adopted.

Oxidation of mixtures of acetaldehydogenic and formaldehydogenic steroids

Preliminary experiments with aqueous solutions of acetaldehyde and formaldehyde showed that a satisfactory separation of the aldehydes at levels of 10 μg. or less was possible under the conditions used for the oxidation and estimation of pregnanetriol. These conditions would be expected to permit quantitative formation of formaldehyde from formaldehydogenic steroids. It was therefore probable that the acetaldehyde and formaldehyde formed on periodate oxidation of mixtures of C_{21} steroids with various types of C-17 side chains could be separated and hence 10–100 μg. amounts of steroids estimated quantitatively.

Table 5. *Recovery of acetaldehyde on oxidation of pregnane-3α:17α:20α-triol by the method finally adopted*

(Oxidation with 0·06 M-HIO_4 in 0·4 N-H_2SO_4.)

Pregnane-triol oxidized (μg.)	Acetaldehyde found (μg.)	Triol equivalent to acetaldehyde found (μg.)	Recovery (%)
58·0	7·38	56·3	97
43·5	5·58	42·6	98
29·0	3·82	29·2	101
17·4	2·16	16·5	95
14·5	1·78	13·6	94

From mixtures of pregnane-3α:17α:20α-triol and 11-deoxycorticosterone or 11-deoxy-17-hydroxycorticosterone the amounts of acetaldehyde and formaldehyde recovered were consistent with the respective amounts of steroids present (Table 6).

Urinary studies

Collection of urine. Urine specimens were collected over 24 hr. with 1–2 ml. $CHCl_3$ as preservative and were analysed as soon after collection as possible.

Preparation of urine extracts. Extracts prepared from unhydrolysed and hydrolysed urine samples have been studied. Two methods have been used for hydrolysis of urine samples. (1) Acidification of urine: a measured portion of 50–100 ml. of a 24 hr. specimen was acidified to pH 0·95–1·1 (glass electrode) with 10 N-H_2SO_4. After 24 hr. at 25° the acidified urine was extracted with $CHCl_3$. For 'hydrolysis curves' larger portions of 24 hr. specimens were acidified to allow a number of samples to be withdrawn for analysis at various time intervals. (2) Incubation of urine with β-glucuronidase preparations: the crude β-glucuronidase was prepared (cf. Kerr & Levvy, 1951) by suspending finely minced fresh ox spleen in acetate buffer, pH 5·2, and standing at 37° for 4 hr. After centrifuging off tissue debris, the supernatant was made 60% saturated with $(NH_4)_2SO_4$. The precipitate was dialysed free of $(NH_4)_2SO_4$, and in further fractionations the material precipitated between 25 and 50% saturated $(NH_4)_2SO_4$ was retained. After dialysis this crude enzyme preparation was assayed, using phenylglucuronide as substrate, and expressing the activity in 'glucuronidase units' (G.U.) where 1 G.U. liberates 1 μg. phenol in 1 hr. under the conditions given by Kerr, Graham & Levvy (1948).

For incubation with enzyme, 25–50 ml. of a 24 hr. urine specimen was acidified to pH 4·5 with acetic acid and 10 ml. 0·1 M-acetate buffer, pH 4·5, added. A solution containing enzyme in the proportion of 10 000 G.U./24 hr. specimen was added and the mixture incubated at 37° for 24 hr. An enzyme blank with 25–50 ml. of water in place of urine was carried out simultaneously. Blank values have been consistently less than 10% of the corresponding estimations.

Neutralization of urine prior to extraction. For comparative purposes it is desirable to extract urine samples after

Table 6. *Recovery of acetaldehyde and formaldehyde from periodate oxidation of mixtures of pregnane-3α:17α:20α-triol (triol), 11-deoxycorticosterone (DOC) and 17-hydroxy-11-deoxycorticosterone (S)*

Steroid oxidized (μg.)			Acetaldehyde found (μg.)	Formaldehyde found (μg.)	Steroids (μg.) equivalent to CH_3CHO and H.CHO found		Steroids oxidized (%)	
Triol	DOC	*S*			Acetaldehydogenic	Formaldehydogenic	Acetaldehydogenic	Formaldehydogenic
58·0	—	36·0	7·42	2·76	56·7	31·9	98	89
34·8	—	36·0	4·50	2·76	34·4	31·9	99	89
11·6	—	36·0	1·60	3·13	12·2	36·1	105	100
52·7	—	54·0	6·05	5·04	46·2	58·1	88	108
31·6	—	54·0	3·96	4·90	30·2	57·0	96	106
21·1	—	54·0	2·88	4·30	21·8	49·6	103	92
10·5	—	54·0	1·40	4·57	10·7	52·8	102	98
52·7	—	72·0	6·66	5·84	50·8	67·4	96	94
31·6	—	72·0	3·49	5·78	26·6	66·7	84	93
21·1	—	72·0	2·97	5·73	22·8	66·1	108	92
52·7	63·2	—	6·33	5·25	48·3	57·7	92	91
31·6	63·2	—	4·05	5·46	30·9	60·0	98	95
10·5	63·2	—	1·47	5·73	11·2	63·0	107	100
52·7	63·2	—	6·75	5·67	51·5	62·4	98	99
31·6	15·8	—	4·50	1·43	34·4	15·7	104	99
21·1	15·8	—	2·70	1·48	20·6	16·3	98	103
10·5	15·8	—	1·35	1·38	10·3	15·2	98	96
52·7	31·6	—	6·75	2·70	51·5	29·7	98	94
31·6	31·6	—	3·96	2·65	30·2	29·2	96	92
10·5	31·6	—	1·37	2·81	10·5	30·9	100	98

various pretreatments under similar conditions. In most experiments reported here, urine samples, prior to extraction, were brought to pH 6·9–7·1 (glass electrode) using 2N-Na_2CO_3 and 0·5M-K_2HPO_4. The samples were stirred vigorously during the addition of alkali.

Extraction of urine. Urine samples were extracted three times with 2 vol. of $CHCl_3$. Using these proportions, emulsions were rarely obtained. The $CHCl_3$ extracts were washed once with 0·1 vol. 0·1N-NaOH and twice with 0·1 vol. water. The water washings were washed once with 2 vol. $CHCl_3$, this $CHCl_3$ being added to the main $CHCl_3$ extract. The $CHCl_3$ extracts were dried with the minimum amount (about 2 g./100 ml.) of anhydrous Na_2SO_4 (A.R.) for 20 min. After filtering off the Na_2SO_4 the $CHCl_3$ extracts were evaporated to dryness under reduced pressure in a water bath at about 60°. The residues were transferred with three 2 ml. portions of redistilled ethanol to the oxidation tubes *A* (Fig. 1). The subsequent procedure is detailed in the Method section (p. 340).

Recovery experiments on urine extracts. The recoveries of pregnane-3α:17α:20α-triol added to $CHCl_3$ extracts of unhydrolysed, acid and enzymically hydrolysed urine were studied, in addition to the recovery of pregnanetriol added to the urine itself (added in 0·5–1·0 ml. ethanol) with subsequent extraction of the urine and purification of extracts. Recoveries were calculated from the increase in acetaldehyde obtained from extracts containing added pregnanetriol over that given by extracts without added triol.

The recoveries of pregnanetriol added to $CHCl_3$ extracts of urine where no alkali washing was carried out, varied from urine to urine (Table 7). With alkali-washed extracts recoveries were consistent, and are illustrated in the results obtained for the recovery of pregnanetriol added to urine itself with subsequent $CHCl_3$ extraction (Table 8).

The urine 'blank' values, i.e. acetaldehyde obtained from urine extracts without added triol, are a measure of the acetaldehydogenic material extractable from urine and may indicate the presence of C_{21} 17:20-dihydroxy-20-methylsteroids. The blank values given in Tables 7 and 8 are expressed as mg. pregnanetriol/24 hr.

In Table 9 are compared 'blank' values obtained from untreated urine, acid hydrolysed and enzymically hydrolysed urine from normal male subjects. The values obtained after acidification or incubation with β-glucuronidase preparations are considerably higher than those from untreated urine (cf. formaldehydogenic steroids in urine (Heard, Sobel & Venning, 1946; Paterson, Cox & Marrian, 1950; Kinsella, Doisy & Glick, 1950; Cox & Marrian, 1951). The amount of acetaldehydogenic material extractable after acidification of urine from normal males increases with time. The increase is rapid for a few hours, after which very little change is observed up to 24 hr. after acidification. ($CHCl_3$

Table 7. *Irregular recovery of pregnanetriol added to unwashed chloroform extracts of normal male urine*

		Pretreatment of urine					
		(1) None		(2) Acid hydrolysis		(3) Enzymic hydrolysis	
	Triol added (μg.)	Triol recovered (%)	Urine 'blank' (mg. as triol/ 24 hr.)	Triol recovered (%)	Urine 'blank' (mg. as triol/ 24 hr.)	Triol recovered (%)	Urine 'blank' (mg. as triol/ 24 hr.)
Urine I	0	—	0·98	—	2·15	—	3·90
	0	—	0·97	—	2·02	—	4·12
	10	19	—	102	—	35	—
	25	8	—	54	—	69	—
	50	62	—	52	—	59	—
Urine II	0	—	0·86	—	2·63	—	3·64
	0	—	0·94	—	2·79	—	3·88
	10	82	—	95	—	117	—
	25	103	—	101	—	90	—
	50	100	—	93	—	77	—

Table 8. *Recovery of pregnanetriol added to urine from normal male subjects. (Chloroform extracts alkali-washed)*

(31·5 μg. triol added to each sample.)

Subject	Urine 'blank' (value as mg. triol/day)	Recovery of added triol (%)
A	0·33	96
	(0·26)*	—
B	0·48	94
	0·49	95
C	0·40	93
	0·40	93
D	0·26	90
	0·29	93

* Loss during processing.

Table 9. *Amounts of acetaldehydogenic material found in urine of normal male subjects*

(All values expressed as mg. pregnanetriol/24 hr.)

Urine sample	Treatment of urine prior to chloroform extraction: Untreated urine	Acidification to pH 1, with immediate extraction	Acidification to pH 1; extracted after 24 hr.	Incubated at 37°, pH 4·5, with β-glucuronidase
	(*A*) Chloroform extracts alkali-washed			
I	0·33	0·44	0·92	—
II	0·28	0·43	—	—
III	0·36	—	—	1·58
IV	0·47	—	—	—
V	0·48	0·93	0·80	1·94
VI	0·12	0·19	0·29	0·66
VII	0·28	0·32	0·45	—
	(*B*) Chloroform extracts not alkali-washed			
VIII	0·92	2·96	2·80	3·60
IX	0·72	3·20	3·40	4·80

extracts were alkali-washed.) As yet, insufficient study has been made of such 'hydrolysis curves' to permit of interpretation, but they indicate the presence in urine of conjugates hydrolysed by acid at 25°. With regard to the acid stability of acetaldehydogenic steroids, it has been found that pregnane-3α:17α:20α-triol is stable to pH 1 at 25° for 24 hr.

DISCUSSION

Estimation of acetaldehyde formed by periodate oxidation of urine extracts is more specific and sensitive than the estimation of 17-ketosteroids formed (Talbot & Eitingon, 1944). As C_{21} 17:20:21-trihydroxysteroids as well as C_{21} 17:20-dihydroxy-20-methylsteroids yield 17-ketosteroids on periodate oxidation, these types of steroids are estimated together in the latter method. It is desirable for metabolic studies that these two types, which include both 21-hydroxy- and 21-deoxy-steroids, should be estimated separately. In addition to the general disadvantages of the 17-ketosteroid methods the Talbot & Eitingon (1944) method estimates the relatively small increase of 17-ketosteroids after periodate oxidation over that present before oxidation.

It has been found that neutral chloroform extracts of normal male urine contain substances which liberate acetaldehyde when oxidized with periodic acid under the same conditions necessary for quantitative oxidation of 17:20-dihydroxy-20-methylsteroids.

The presence of some types of conjugates was indicated by the increased amounts of acetaldehydogenic material extractable from urine after treatment with acid or β-glucuronidase preparations. Although C_{21} 17:20-dihydroxy-20-methylsteroids have been isolated from urine of patients with hyperactive adrenals, proof of the presence of such compounds in normal urine must await isolation studies. This work is now in progress.

Some of the hydrolysis techniques used in the study of urinary formaldehydogenic steroids have been applied to the study of acetaldehydogenic substances. These relatively mild hydrolysis conditions were employed to avoid excessive destruction of the sensitive C-17 side chain (in particular the tertiary hydroxyl at C-17). The value of enzymic hydrolysis with β-glucuronidase (especially when carried out on urine directly) for quantitative assay of urinary cortical steroids or their metabolites seems doubtful at present, when there is not adequate evidence that such enzymic hydrolysis is complete.

Acetaldehyde gives a brown coloration with chromotropic acid (McFadyen, 1945) and may interfere in the estimation of formaldehyde if present in relative excess over the latter. Where estimation of the acetaldehyde is not desired, it may be removed by aeration from the formaldehyde distillates in the Daughaday (1948) method if 1% aqueous glycine is used in place of the ethanolic sulphite solution.

SUMMARY

1. A method for estimating C_{21} 17:20-dihydroxy-20-methylsteroids in urine, based on periodic acid oxidation, is described.

2. The method allows of the simultaneous estimation of acetaldehydogenic and formaldehydogenic steroids.

3. A urinary excretion of unconjugated acetaldehydogenic material equivalent to 0·1–0·5 mg./day of C_{21} 17:20-dihydroxy-20-methylsteroids was found for seven normal males.

4. The presence of conjugated acetaldehydogenic material in normal male urine is also indicated.

The author sincerely thanks Prof. G. F. Marrian, F.R.S., for his constant encouragement and guidance throughout this work, and for his generous gift of pregnane-3α:17α:20α-triol. Part of the expenses of this work were defrayed from Medical Research Council funds.

REFERENCES

Barker, S. B. & Summerson, W. H. (1941). *J. biol. Chem.* **138**, 535.
Butler, G. C. & Marrian, G. F. (1937). *J. biol. Chem.* **119**, 565.
Butler, G. C. & Marrian, G. F. (1938). *J. biol. Chem.* **124**, 237.
Corcoran, A. C. & Page, I. H. (1948). *J. Lab. clin. Med.* **33**, 1326.
Cox, R. I. & Marrian, G. F. (1951). *Biochem. J.* **48**, xxxiii.
Cuyler, W. K., Ashley, C. & Hamblen, E. C. (1940). *Endocrinology*, **27**, 177.
Daughaday, W. H., Jaffe, H. & Williams, R. H. (1948). *J. clin. Endocrinol.* **8**, 166.
Heard, R. D. H., Sobel, H. & Venning, E. H. (1946). *J. biol. Chem.* **165**, 699.
Hechter, O. (1950). *Fed. Proc.* **9**, 58.
Hirschmann, H. & Hirschmann, F. B. (1947). *J. biol. Chem.* **167**, 7.
Hirschmann, H. & Hirschmann, F. B. (1950). *J. biol. Chem.* **187**, 137.
Hollander, V. P., Di Mauro, S. & Pearson, O. H. (1951). *Endocrinology*, **49**, 617.
Horwitt, B. N., Dorfman, R. I., Shipley, R. A. & Fish, W. R. (1944). *J. biol. Chem.* **155**, 213.
Karunairatnam, M. C. & Levvy, G. A. (1949). *Biochem. J.* **44**, 599.
Kerr, L. M. H., Graham, A. F. & Levvy, G. A. (1948). *Biochem. J.* **42**, 191.
Kerr, L. M. H. & Levvy, G. A. (1951). *Biochem. J.* **48**, 209.
Kinsella, R. A., Doisy, R. J. & Glick, J. H. (1950). *Fed. Proc.* **9**, 190.
Koenemann, R. H. (1940). *J. biol. Chem.* **135**, 105.
Lieberman, S. & Dobriner, K. (1945). *J. biol. Chem.* **161**, 269.
Lieberman, S., Fukushima, D. K. & Dobriner, K. (1950). *J. biol. Chem.* **182**, 299.
Lieberman, S., Hariton, L. B., Stockem, M. B., Studer, P. E. & Dobriner, K. (1951). *Fed. Proc.* **10**, 216.
Lowenstein, B. E., Corcoran, A. C. & Page, I. H. (1946). *Endocrinology*, **39**, 82.
McFadyen, D. A. (1945). *J. biol. Chem.* **158**, 107.
Marrian, G. F. (1951). *J. Endocrinol.* **7**, lxix.
Mason, H. L. (1948). *J. biol. Chem.* **172**, 783.
Mason, H. L. & Kepler, E. J. (1945). *J. biol. Chem.* **161**, 235.
Miller, A. M. & Dorfman, R. I. (1950). *Endocrinology*, **46**, 6.
Miller, B. F. & Muntz, J. A. (1938). *J. biol. Chem.* **126**, 413.
Paterson, J. Y. F., Cox, R. I. & Marrian, G. F. (1950). *Biochem. J.* **46**, xxix.
Pincus, G., Hechter, O. & Zaffaroni, A. J. (1951). *Second Conference on Clinical ACTH*, vol. I. London: Churchill.
Reich, H., Nelson, D. H. & Zaffaroni, A. J. (1950). *J. biol. Chem.* **187**, 411.
Russell, J. A. (1944). *J. biol. Chem.* **156**, 463.
Shinn, L. A. & Nicolet, B. H. (1941). *J. biol. Chem.* **138**, 91.
Talbot, N. B. & Eitingon, I. V. (1944). *J. biol. Chem.* **154**, 605.

The Metabolism of Subcutaneously Injected [^{15}N] Urea in the Cat

By H. L. KORNBERG and R. E. DAVIES

Medical Research Council Unit for Research in Cell Metabolism, Department of Biochemistry, The University, Sheffield 10

(*Received* 29 *February* 1952)

The experiments described in this paper arose from a study of the physiological role of gastric urease. In order to throw light on this problem, an investigation was undertaken into the fate of [^{15}N]urea in the cat.

Although Luck (1924) demonstrated the presence of urease in the stomach, urea has been widely regarded solely as an end product of mammalian nitrogen metabolism. This view was supported by experiments of Schoenheimer (1942) and Bloch (1946) who fed [^{15}N]urea to rats and found no more than a slight incorporation of ^{15}N into the proteins of liver and intestine. They believed that this could be interpreted as the result of bacterial decomposition of urea in the gut. More recently, Leifer, Roth & Hempelmann (1948) injected [^{14}C]urea into mice and recovered 20·8% of the injected ^{14}C in the carbon dioxide of the expired air. The breakdown of [^{14}C]urea in mice has been confirmed by Jones (1950) and by Skipper *et al.* (1951). Kornberg, Davies & Wood (1952) made analogous observations on cats.

In the study of the metabolism of [^{15}N]urea it must be borne in mind that the ammonia produced by hydrolysis of urea is largely reconverted to urea in the liver. Hence the amounts of ^{15}N incorporated into other nitrogenous materials give only minimum values for the urea breakdown. In order to arrive at an estimate of the actual breakdown, information is required on the conversion of [^{15}N]ammonia to [^{15}N]urea under the conditions of the experiment.

Although previous workers have studied the fate in the animal body of [^{15}N]ammonia after oral administration of [^{15}N]ammonium citrate (Foster, Schoenheimer & Rittenberg, 1939; Rittenberg, Schoenheimer & Keston, 1939; Sprinson, 1948; Sprinson & Rittenberg, 1949), and after intravenous injection of a single dose of [^{15}N]ammonium glutamate (Berenbom & White, 1950), their results did

not supply the required information. The behaviour of [^{15}N]ammonia liberated slowly and continuously into the blood was therefore investigated after periodic subcutaneous injections of [^{15}N]ammonium lactate into a cat. The amounts of urea hydrolysed, as determined by this procedure, agree with those found subsequently after injection of [^{14}C]urea into cats (Kornberg *et al.* 1952) and are far less than those found in mice (Leifer *et al.* 1948; Skipper *et al.* 1951). Part of this work has been communicated to the Biochemical Society (Davies & Kornberg, 1950).

METHODS

Preparation of labelled materials. All labelled [^{15}N] compounds administered contained 32 atoms % excess ^{15}N in the positions labelled. [^{15}N]Urea was synthesized according to Cavalieri, Blair & Brown (1948) by the interaction of [^{15}N]NH_4NO_3 (the nitrate ion being unlabelled) and diphenyl carbonate, prepared according to Hentschel (1884). The 0·31 M-[^{15}N]ammonium lactate solution was obtained by distilling [^{15}N]NH_3 from [^{15}N]NH_4NO_3 into excess lactic acid (British Pharmacopœia) and neutralizing the solution with M-$NaHCO_3$ to pH 6·5.

Treatment of cats. Cats were fed on bread, milk and water for 4 days prior to, and during, the experiment. Into cat *A* (2·00 kg.) were injected subcutaneously 98·8 mg. [^{15}N]urea in 2 ml. of water the first day, and 116·4 mg. [^{15}N]urea in 2 ml. of water the second day. The total quantity of isotope injected was 32·1 mg. ^{15}N. Cat *B* (1·20 kg.) received 28 mg. ^{15}N in a total of ten subcutaneous injections, at 4-hourly intervals, each of 2 ml. of the [^{15}N]ammonium lactate solution. Urine was collected in flasks containing 10 ml. of 10 N-HCl and 2 ml. of toluene to prevent bacterial decomposition of the constituents and loss of NH_3. On the third day of the experiment, the cats were weighed, anaesthetized with ether, and bled from the carotid arteries. The blood was collected and stored at −12°. The heads were cut off and dropped into liquid N_2; they were removed after 2–3 min. and, on being tapped gently with a hammer, split neatly along the sagittal lines. The halves were stored at −12°. The liver, spleen, kidneys, diaphragm, heart, and pieces of skin and hair were removed from each cat and frozen rapidly in solid CO_2. The stomach and intestinal tract of each animal were opened and washed under the tap before being frozen. All tissues were stored at −12°.

Analysis of urine samples. Samples (3 ml.) were pipetted into all-glass aeration units (Van Slyke & Cullen, 1916), 3 ml. of sat. K_2CO_3 were added and a rapid stream of air passed for 3 hr., to transfer the NH_3 to 0·05 N-H_2SO_4 (5 ml.). The absorbed NH_3 was estimated by titration with 0·05 N-NaOH using bromocresol green as indicator. When the end points had been reached, the NH_3 solutions were immediately acidified with 3 N-HCl and evaporated on an electric hot-plate to approx. 1 ml. They were then transferred to small ampoules which were sealed and sent off for mass spectrometric analysis.

This procedure removed 96–98 % of the [^{15}N]NH_3 present. The remainder was washed out by addition of 1 ml. of 0·214 M-NH_4Cl (containing 3 mg. N) and 1 ml. of 40 % (w/v) NaOH to the solutions, and aerating the NH_3 for 2 hr. into 0·05 N-H_2SO_4 (5 ml.). This was repeated once more: the last NH_3 samples thus obtained were always free from excess ^{15}N.

The alkaline urine samples, which were now NH_3-free, were transferred to measuring cylinders. Approximately half of the solutions were pipetted into 250 ml. beakers and neutralized to thymol blue by dropwise addition of 10 N-HCl. The urea present in each solution was then precipitated as the dixanthydryl complex (Fosse, 1916; Kiech & Luck, 1928), filtered off on weighed Royal Worcester 1/3114 filter crucibles, washed and air-dried at 110° for 3 hr. Samples (20–30 mg.) were digested by the Kjeldahl procedure. The NH_3 formed was distilled into 5 ml. of 0·05 N-HCl (Rittenberg, 1946), estimated by titration against 0·05 N-NaOH and prepared for mass spectrometric analysis as described above.

This procedure removed 95–98 % of the [^{15}N]urea present. The remainder was washed out by twice adding 2 ml. of 20 % urea to the urine samples and isolating the dixanthydryl complex. Kjeldahl and mass spectrometric analysis of the last urea samples thus obtained always showed their N to be present in the normal ratio of isotope abundance. The remaining solutions, which were now free of both [^{15}N]NH_3 and [^{15}N]urea, were digested by the Kjeldahl procedure. The NH_3 formed was estimated and prepared for mass spectrometric analysis as described above. No attempts were made to isolate the various constituents of these solutions, the N of which will be referred to as the non-urea non-ammonia N.

Independent checks of the results for the various urine fractions were obtained in all cases by a second procedure. In this, duplicate samples of 0·5–1·0 ml. of urine were pipetted into aeration units, the acid neutralized by dropwise addition of M-$NaHCO_3$ and the pH adjusted to 5·0 by addition of 0·5 ml. of 3 M-acetate, pH 5·0. Water (2 ml.) and jack bean urease solution (1 ml.), prepared as described by Davies & Kornberg (1951), were added to hydrolyse the urea, and the units left overnight. Saturated K_2CO_3 (3 ml.) was added and the NH_3 aerated into 0·05 N-H_2SO_4 (15 ml.) for 3 hr. This removed 96–98 % of the [^{15}N] (NH_3 + urea) N present in the original samples. The remainder was washed out by addition of 1 ml. of 0·214 M-NH_4Cl (containing 3 mg. N) and 1 ml. of 40 % (w/v) NaOH and drawing off the NH_3 for 2 hr. into 5 ml. of 0·05 N-H_2SO_4. This was repeated once more: the last NH_3 samples thus obtained were always found to contain N with normal isotope abundance ratio. The liquids remaining in the units, which were now free of both NH_3 and urea, were washed into Kjeldahl flasks and digested by the Kjeldahl procedure. The ammonia formed was estimated and prepared for mass-spectrometric analysis as described above, this fraction being the non-urea non-ammonia N. The two methods gave results for the isotope distribution in the various fractions of urine which agreed within 5 %.

Analysis of tissue samples. Portions (50–100 mg.) of the tissues were digested by the Kjeldahl procedure. The NH_3 formed (total N) was estimated and prepared for mass spectrometric analysis as described previously.

The liver of each cat was homogenized with 5 % trichloroacetic acid and centrifuged at 3000 rev./min. for 30 min. Portions of the supernatant liquid containing the N.P.N. were digested by the Kjeldahl procedure. The precipitate was twice washed with equal volumes of 5 % trichloroacetic acid, spun down and oven dried. The protein precipitate was boiled under reflux for 20 hr. with 10 vol. of 5 N-HCl and filtered. Excess MgO was added to portions of the filtrate, containing the amide N, and the NH_3 was drawn into 0·05 N-H_2SO_4. The solutions from which the amide N had been removed were washed twice by adding 1 ml. of 0·214 M-NH_4Cl (containing 3 mg. N) and 1 ml. 40 % (w/v)

NaOH, and aerating the NH_3 into 0·05 N-H_2SO_4. Portions of the remaining solutions, containing the non-ammonia non-amide fractions of the protein N, were digested by the Kjeldahl procedure, estimated and prepared for mass spectrometric analysis as above.

^{15}N determinations. All samples for isotopic analysis contained 1–3 mg. N as NH_4^+ salts in approx. 1 ml. of solution. Their ^{15}N contents were determined by Dr R. Bentley and Mr G. Dickinson with the mass spectrometer of the National Institute for Medical Research, Mill Hill, London.

RESULTS

The breakdown of [^{15}N]urea

The results obtained after subcutaneous injection of [^{15}N]urea into cat *A* are given in Table 1. Most of the isotope injected (83·5 %) was excreted in the urine, 94·8 % of this as [^{15}N]urea. The 16·5 % of injected isotope still remaining in the body were widely distributed in all the tissues and body fluids examined. From the concentration and isotope content of blood urea, and from the fact that the 'urea space' of the cat (i.e. the volume of fluid in which the urea is uniformly distributed), is 65 % of the body weight of the animal (Eggleton, 1951; Kornberg *et al.* 1951; Kornberg, Davies & Wood, 1952), it was calculated that not more than 2·6 % of the ^{15}N injected was present in the body as [^{15}N] urea. Over a half of the remaining 13·9 % of isotope was incorporated into muscle, for, although the isotope abundance ratio of muscle nitrogen was only slightly in excess of normal, this tissue forms quantitatively the major nitrogenous component of the body. The highest enrichments with ^{15}N of tissue nitrogen were found in liver, small intestine, kidney and heart muscle. The liver amide nitrogen contained 0·013 atom % excess ^{15}N. These results show that the [^{15}N]urea injected into the cat had been broken down in the body. This conclusion is also indicated by the distribution of isotope in the various constituent fractions of the urine. Although over 94 % of the isotope present in the urine was in the form of [^{15}N]urea, 0·2 % of the ^{15}N was found in

Table 1. *Distribution of ^{15}N after subcutaneous injection of [^{15}N]urea into a cat*

(Cat *A*, 2·0 kg. body wt., received 215 mg. [^{15}N]urea (containing 32 atoms % excess ^{15}N, i.e. 32·1 mg. ^{15}N) in two doses: 98·8 mg. the first day, 116·4 mg. the second. Accuracy of mass spectrometer; ±0·003 atoms % excess ^{15}N.)

Material examined	Total N content (g.)	Total ^{15}N content (atom % excess)	Total ^{15}N content (mg. ^{15}N)	Percentage of injected ^{15}N
Urine, total N	2·50	1·075	26·8	83·5
Urine, urea N	1·83	1·287	25·4	79·1
Urine, ammonia N	0·13	0·042	0·054	0·17
Urine, non-urea non-ammonia N	0·55	0·136	0·72	2·3
Body, urea N	0·39	0·210	0·82	2·6
Skeletal muscle, total N	46·5	0·005	2	7
Skin and hair, total N	14	0·004	0·6	2
Liver, total N	1·66	0·008	0·13	0·4
Liver, amide N	0·08	0·013	0·01	0·04
Small intestine, total N	2·72	0·009	0·25	0·8
Gastric mucosa, total N	0·15	0·003	0·004	0·01
Heart muscle, total N	0·254	0·007	0·018	0·06
Kidneys, total N	0·465	0·008	0·04	0·1
Total ^{15}N found in fractions analysed	—	—	30·7	96

Table 2. *Distribution of ^{15}N after subcutaneous injection of [^{15}N]ammonium lactate into a cat*

(Cat *B*, 1·2 kg. body wt., received 20 ml. 0·31 M-[^{15}N]ammonium lactate (containing 32 atoms % excess ^{15}N, i.e. 28·0 mg. ^{15}N) in ten doses at 4-hourly intervals. Accuracy of mass spectrometer: ±0·003 atoms % excess ^{15}N.)

Material examined	Total N content (g.)	Total ^{15}N content (atom % excess)	Total ^{15}N content (mg. ^{15}N)	Percentage of injected ^{15}N
Urine, total N	—	—	15·48	55·4
Urine, urea N	—	—	12·80	45·7
Urine, ammonia N	—	—	1·54	5·5
Urine, non-urea non-ammonia N	—	—	0·86	3·1
Body, urea N	0·22	1·086	2·38	8·5
Skeletal muscle, total N	29·8	0·016	4·8	17
Skin and hair, total N	12	0·008	1·0	4
Liver, total N	1·33	0·069	0·98	3·5
Liver, amide N	0·07	0·415	0·27	0·96
Small intestine, total N	1·68	0·082	1·4	5·0
Gastric mucosa, total N	0·10	0·016	0·016	0·06
Heart muscle, total N	0·17	0·034	0·06	0·22
Kidneys, total N	0·26	0·067	0·17	0·68
Total ^{15}N found in fractions analysed	—	—	26·3	94

the ammonia and over 2·3 % as non-urea non-ammonia nitrogen. The nitrogen of the gastric mucosa was not significantly enriched with ^{15}N, which suggests that the breakdown of [^{15}N]urea by the body was not connected with any local synthesis (e.g. of mucus) in this tissue.

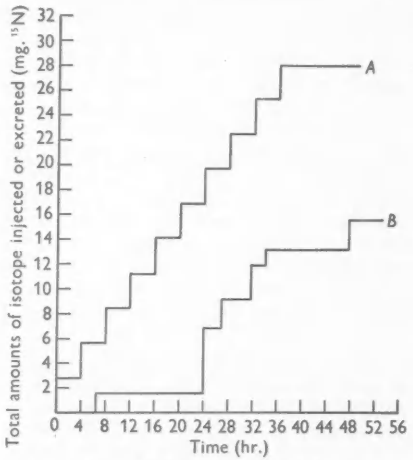


Fig. 1. Excretion of ^{15}N following periodic subcutaneous injection of [^{15}N]ammonium lactate into a cat. Curve *A* shows the amounts of ^{15}N injected (2·8 mg. ^{15}N every 4 hr.), curve *B* shows the total ^{15}N excreted in the urine.

The behaviour of [^{15}N] *ammonia in the body*

When [^{15}N]ammonium lactate was injected subcutaneously at 4-hourly intervals over 40 hr. into cat *B*, 55·4 % of the injected ^{15}N was excreted in the urine (Table 2). The rate of excretion paralleled the rate of administration of the isotope (Fig. 1), showing that after two or three injections the ^{15}N had equilibrated with the body nitrogen. The distribution of the isotope among the various fractions of the urine samples analysed (Table 3) shows that most of the isotope was excreted in the form of [^{15}N]urea, but that considerable and variable proportions appeared as ammonia nitrogen and non-urea non-ammonia nitrogen. The ^{15}N content of the the ammonia nitrogen was higher than that of the non-urea non-ammonia nitrogen in all samples except urine (*b*) which had not been voided until nearly 18 hr. after the previous sample.

The results given in Table 2 show that of the ^{15}N injected as [^{15}N]ammonia after 40 hr. 8·5 % was present in the body as [^{15}N]urea. The remaining 36 % had been incorporated into the tissues, over 47 % of this residue being present in muscle. The highest enrichments of tissue nitrogen were found, as after injection of [^{15}N]urea, in liver, small intestine, kidney and heart muscle, all these tissues having high rates of metabolic activity. The isotope abundance ratio of the nitrogen of gastric mucosa was similar to that of skeletal muscle, again showing that ammonia nitrogen was not preferentially incorporated in this tissue by local synthetic mechanisms.

DISCUSSION

The breakdown of urea

The slight incorporation of ^{15}N into tissue proteins, reported by Schoenheimer (1942) and Bloch (1946) after feeding [^{15}N]urea to rats, was regarded by these authors as the result of bacterial decomposition of urea in the gut and was not considered evidence for the view that urea is metabolized by the animal.

In the present experiments on the fate of [^{15}N]urea in the body, cats were used as experimental animals because the gastric urease content of the ground stomach of cats is much higher than that of rats (Luck, 1924; FitzGerald, 1946). The risk of bacterial decomposition of isotopic materials in the gut was reduced by administering the compounds by subcutaneous injection. The results obtained after injecting [^{15}N]urea into cat *A* (Table 1) show that after 40 hr. at least 2·5 % of the [^{15}N]urea had been broken down in the body. This figure represents a minimum, since the [^{15}N]ammonia formed by hydrolysis of [^{15}N]urea would be largely reconverted to [^{15}N]urea in the liver. Quantitative estimates of the amounts of urea breakdown can be made only if the behaviour of [^{15}N]ammonia in the

Table 3. *Distribution of* ^{15}N *in urine samples from a cat after subcutaneous injection of* [^{15}N]*ammonium lactate*

(Cat *B*, 1·2 kg. body wt., received 20 ml. 0·31 M-[^{15}N]ammonium lactate (containing 32 atoms % excess ^{15}N, i.e. 28·0 mg. ^{15}N) in ten doses at 4-hourly intervals. Six urine samples (*a*–*f*) were voided by the cat and were collected in flasks containing 10 ml. of 10 N-HCl and 2 ml. of toluene.)

Fraction	^{15}N content of urine samples (mg. ^{15}N)					
	(*a*)	(*b*)	(*c*)	(*d*)	(*e*)	(*f*)
Total N	1·58	5·25	2·33	2·76	1·24	2·32
Ammonia N	0·197	0·254	0·247	0·300	0·245	0·297
Urea N	1·30	4·32	1·98	2·33	0·96	1·91
Non-urea non-ammonia N	0·060	0·620	0·049	0·065	0·017	0·050
Recovery (%)	99	99	98	98	99	98

body is known. The results obtained with cat *B* show that in 40 hr. 54% of the ammonia produced by hydrolysis of urea was converted to urea and therefore the value of at least 2·5% breakdown found with cat *A* actually represents a breakdown of at least 5%.

A similar figure is indicated by the amounts of incorporation of ^{15}N into liver amide nitrogen in the two experiments. After injection of [^{15}N]ammonia, the liver amide nitrogen contained isotope at 0·415 atoms % excess of normal, whilst after [^{15}N]urea injection the isotope abundance ratio of this fraction was 0·013 atoms % excess. The amount of isotope injected in the two cats, expressed as mg. ^{15}N/kg. body weight, was 1·45 times as much in cat *B* as it was in cat *A*, and for equivalent amounts of isotope injected the liver amide nitrogen of cat *B* would thus have contained of the order of 0·28 atoms % excess ^{15}N. This would indicate that at least 4·6% of the [^{15}N]urea injected into cat *A* had been broken down to [^{15}N]ammonia in the body.

Preliminary studies on the breakdown of [^{14}C]urea in anaesthetized cats (Kornberg *et al.* 1952) indicated an actual rate of breakdown of approx. 1%/5 hr., i.e. approx. 8% over 40 hr. The values obtained in the present experiments are therefore in good agreement with those obtained with [^{14}C]urea, and are much lower than those reported for the breakdown of urea in mice (Leifer *et al.* 1948; Jones, 1950; Skipper *et al.* 1951).

The role of gastric urease

It has frequently been suggested that gastric urease, by hydrolysing urea to ammonium carbonate, supplies base which might protect the mucosa from attack by acid and pepsin (Luck & Seth, 1925; Linderstrøm-Lang & Ohlsen, 1936; Ohlsen, 1941; FitzGerald, 1946; Fossel, 1947; FitzGerald & Murphy, 1950; Glick, Zak & Korff, 1950; Von Korff, Ferguson & Glick, 1951). The total urease content of the cat's stomach has been claimed to be sufficiently high to liberate from urea 66 mg. ammonia nitrogen/hr. (FitzGerald, 1946), i.e. sufficient to break down the total urea content of a 2 kg. cat every 7 hr. The present results indicate that the actual urea breakdown in the intact cat occurs at less than one-hundredth of this rate.

It has been suggested (FitzGerald & Murphy, 1950) that the ammonia set free by the breakdown of urea is transformed inside the mucosa into some product of low toxicity, which has not been identified, but which is not thought to be urea. The present results, although they indicate neither the site nor the significance of the breakdown of urea, show that gastric urease plays no part in such local synthetic mechanisms.

SUMMARY

1. When [^{15}N]urea was injected subcutaneously into a 2·0 kg. cat, 2·5% of the ^{15}N was excreted in the urine in forms other than urea. The tissues contained nitrogen with slight but significant excess of isotope.

2. The fate of [^{15}N]ammonia in the body was studied by measuring the distribution of ^{15}N in the tissues and urine after subcutaneous injection into a 1·2 kg. cat of [^{15}N]ammonium lactate in ten 4-hourly doses. Of the nitrogen of the injected [^{15}N]ammonium lactate 54·2% was converted to [^{15}N]urea. It follows that in 40 hr., at least 5% of the urea of the cat was broken down in the body.

3. After administration of [^{15}N]urea or [^{15}N]ammonium lactate the nitrogen of the gastric mucosa was not preferentially enriched with ^{15}N; this argues against the participation of gastric urease in local synthetic mechanisms.

We wish to thank Prof. H. A. Krebs, F.R.S., for his encouragement and interest in this work. One of us (H.L.K.) wishes to acknowledge the receipt of a grant from the Medical Research Council during part of this work, the remainder being done during the tenure of the John Stokes Research Fellowship of the University of Sheffield.

REFERENCES

Berenbom, M. & White, J. (1950). *J. biol. Chem.* **182**, 5.
Bloch, K. (1946). *J. biol. Chem.* **165**, 469.
Cavalieri, L. F., Blair, V. E. & Brown, G. B. (1948). *J. Amer. chem. Soc.* **70**, 1240.
Davies, R. E. & Kornberg, H. L. (1950). *Biochem. J.* **47**, viii.
Davies, R. E. & Kornberg, H. L. (1951). *Biochem. J.* **50**, 119.
Eggleton, M. G. (1951). *J. Physiol.* **115**, 482.
FitzGerald, O. (1946). *Nature, Lond.*, **158**, 305.
FitzGerald, O. & Murphy, P. (1950). *Irish J. med. Sci.* p. 97.
Fosse, R. (1916). *Ann. Chim. Phys.* **6**, 13.
Fossel, M. (1947). *Hoppe-Seyl. Z.* **282**, 164.
Foster, G. L., Schoenheimer, R. & Rittenberg, D. (1939). *J. biol. Chem.* **127**, 319.
Glick, D., Zak, E. & Von Korff, R. (1950). *Amer. J. Physiol.* **163**, 386.
Hentschel, W. (1884). *Ber. dtsch. chem. Ges.* **17**, 1287.
Jones, H. B. (1950). Personal communication.
Kiech, V. C. & Luck, J. M. (1928). *J. biol. Chem.* **77**, 723.
Kornberg, H. L., Davies, R. E. & Wood, D. R. (1952). *Proc. Isotopes Techniques Conf.* (in the Press).
Kornberg, H. L., Davies, R. E. & Wood, D. R. (1952). *Nature, Lond.*, **169**, 502.
Leifer, E., Roth, L. J. & Hempelmann, L. H. (1948). *Science*, **108**, 748.
Linderstrøm-Lang, K. & Ohlsen, A. S. (1936). *Enzymologia*, **1**, 92.

Luck, J. M. (1924). *Biochem. J.* **18**, 825.
Luck, J. M. & Seth, T. N. (1925). *Biochem. J.* **19**, 357.
Ohlsen, A. S. (1941). *C.R. Lab. Carlsberg, Ser. chim.* **23**, 329.
Rittenberg, D. (1946). In *Preparation and Measurement of Isotopic Tracers*. Ann Arbor, Michigan: J. W. Edwards.
Rittenberg, D., Schoenheimer, R. & Keston, A. S. (1939). *J. biol. Chem.* **128**, 603.
Schoenheimer, R. (1942). *The Dynamic State of Body Constituents*. Cambridge, Mass.: Harvard University Press.
Skipper, H. E., Bennett, L. L. jun., Bryan, C. E., White, L. jun., Newton, M. A. & Simpson, L. (1951). *Cancer Res.* **11**, 46.
Sprinson, D. B. (1948). In *A Symposium on the Use of Isotopes in Biology and Medicine*. Madison: University of Wisconsin Press.
Sprinson, D. B. & Rittenberg, D. (1949). *J. biol. Chem.* **180**, 707.
Van Slyke, D. D. & Cullen, G. E. (1916). *J. biol. Chem.* **24**, 117.
Von Korff, R., Ferguson, D. J. & Glick, D. (1951). *Amer. J. Physiol.* **165**, 695.

The Determination of Basic Groups in Collagen and Gelatin

By S. C. Ellis and K. G. A. Pankhurst

The British Leather Manufacturers' Research Association, Milton Park, Egham, Surrey

(*Received* 26 *January* 1952)

Arising out of a study of the changes taking place in solutions of collagen and gelatin in organic solvents such as formic acid, it became necessary to devise a quick method of estimating the basic groups of the protein. The standard methods, such as determination of the amount of acid bound in aqueous solution, amino-acid analysis, or the adsorption of anionic dyes (Fraenkel-Conrat & Cooper, 1944) or other surface active agents (Pankhurst, 1949), were not suitable, since, besides being time-consuming, they necessitate removal of the organic solvent before making the determination. This would almost certainly introduce additional changes in the protein molecule.

Harris (1935) has shown that the amino groups in amino-acids could be determined directly by titration with perchloric acid, using anhydrous acetic acid as solvent, the amino-acid behaving as a strong base which titrates with a sharp colorimetric end point. The reactions may be formulated thus:

$$R.NH_2.COOH + CH_3COOH = R.NH_3^+.COOH + CH_3COO^-, \quad (1)$$

$$HClO_4 + CH_3COOH = CH_3COOH_2^+ + ClO_4^-, \quad (2)$$

$$CH_3COOH_2^+ + CH_3COO^- = 2CH_3COOH. \quad (3)$$

(Cf. $H_3O^+ + OH^- = 2H_2O$.)

Equation 1 proceeds from left to right since the amino-acid is more protophilic than the acetic acid. In equation 2 the perchloric acid being strongly protogenic causes the acetic acid to behave as a base. Equation 3 represents the neutralization of the negatively charged acetate ion by the $CH_3COOH_2^+$ ion (cf. the hydrogen ion H_3O^+).

The detection of this end point using an indicator such as brilliant cresyl blue is an easy matter with amino-acids and simple peptides and can be carried out when either acetic acid or formic acid is used as solvent, although with the latter the end point is a little less sharp. With gelatin and collagen solutions, however, the method was not successful since a precipitate formed during the course of the titration, on to which the indicator became adsorbed. In view of this complication electrical methods of determining the end point were investigated.

EXPERIMENTAL AND RESULTS

Potentiometric method

The quinhydrone electrode can frequently be used for pH titrations in aqueous solutions, since the redox potential is dependent on pH. The use of quinhydrone in non-aqueous systems is made difficult by its relatively high solubility. Hall & Conant (1927) have overcome this by using 'chloranil' (tetrachloroquinone). Using the apparatus shown diagrammatically in Fig. 1 we have used this method for glycine and gelatin and have obtained the results shown in Fig. 2. To carry out the titration, approx. 0·1 g. of glycine or 1 g. of gelatin was dissolved in approx. 100 ml. of anhydrous acetic acid, or where necessary in a mixture of anhydrous formic and acetic acids (1:4 by volume), and saturated with an equimolecular mixture of chloranil and hydrochloranil in the reaction flask. Normally, two electrodes of brightly polished platinum were used and the potential difference between each and a calomel half-cell was measured; this provides a check against possible insensitivity due to faulty preparation or poisoning of the electrodes. The calomel half-cell, used as reference, was connected to the reaction chamber by a salt

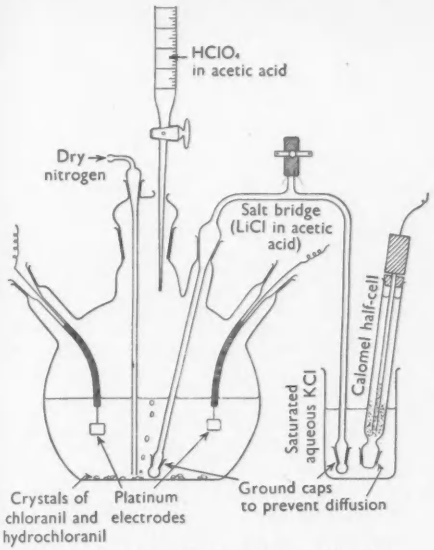


Fig. 1. Apparatus for potentiometric titration.

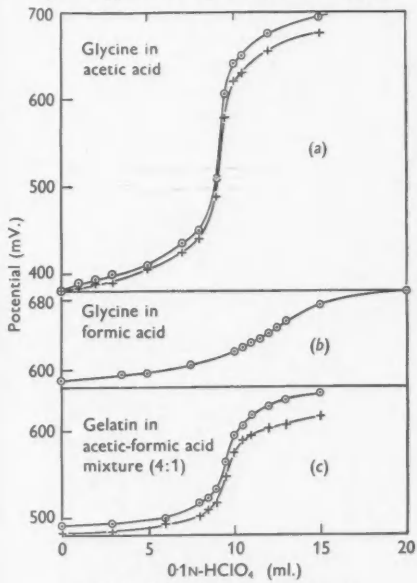


Fig. 2. Potentiometric titrations.

bridge containing a saturated solution of lithium chloride in anhydrous acetic acid, dipping into a saturated aqueous solution of potassium chloride. Moisture was excluded and stirring effected by a stream of dry nitrogen passing through the reaction mixture.

Fig. 2*a* shows that glycine dissolved in acetic acid titrates as a typical strong base and the end point is easily determined. Glycine dissolved in formic acid (Fig. 2*b*) gives a curve with a very gentle slope from which the end point cannot accurately be found. This may seem surprising since formic acid is more protogenic than acetic acid and it would therefore be expected that even stronger basic characteristics would be exhibited by the glycine. However, the absolute value of the potential is dependent on the solvent, for example, the initial potential in formic acid is over 200 mV. higher than in acetic acid. Since the titrating solution is made up in acetic acid, the solvent mixture continuously changes as the titration progresses, becoming increasingly rich in acetic acid, which has the opposite effect on the potential compared with the neutralization process.

For the titration of gelatin a mixture of acetic and formic acids containing not more than 20 % (v/v) of formic acid can be used, as seen in Fig. 2*c*, although even this amount of formic acid causes an increase in initial potential by about 100 mV., thereby reducing the total change.

Conductometric method

Formic acid was essential for the work on collagen and gelatin, and in view of its adverse effect on the potentiometric titrations, even when used only to the extent of 20 % (v/v) in admixture with acetic acid, a conductometric method was investigated. Using an orthodox conductivity apparatus (cell capacity approx. 110 ml. and cell constant 0·3485) it was found that satisfactory end points were obtained with solutions of glycine, gelatin and collagen in mixtures of formic and acetic acids (1:4 by volume). A typical result for collagen is shown in Fig. 3. For this method approx 0·1 g. of amino-acid or 1 g. of protein is dissolved in 50 ml. of the mixed solvent.

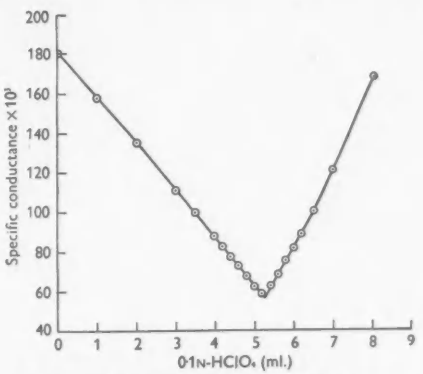


Fig. 3. Conductometric titration of collagen.

Preparation of standard perchloric acid

Aqueous perchloric acid (AnalaR; 60 % w/v) was standardized by titration against sodium hydroxide, and an accurately weighed amount of acetic anhydride equivalent to the water present was slowly added dropwise with ice-cooling. All the water having thus been converted to acetic acid, further anhydrous acetic acid was added to produce a 0·1 M solution. The solution, which is extremely hygroscopic, must be kept in an automatic burette filler from which moisture is excluded. If the quantities of standardized aqueous perchloric acid and acetic anhydride are accurately weighed, there is no need for further standardization and, provided moisture is excluded, the solution keeps well for at least a year. The standardization may be checked from time to time by titration against AnalaR glycine, using the colorimetric method of Harris (1935).

CONCLUSIONS

Of the three methods tested, only the potentiometric and conductometric end points are suitable for use with collagen and gelatin. The conductometric method is the easier to perform, although both electrical methods give results similar to those obtained with other methods, as shown in Table 1.

Any increase in basic groups brought about by the dissolution of the protein in formic acid at 35°, such as was detected in collagen by Bowes & Moss (1951) using Sanger's (1945) method of end-group analysis, would not be detected by this method as it would be within the limit of experimental error.

Table 1. *Basic groups of collagen and gelatin*

	Collagen (m-equiv./g.)	Gelatin (m-equiv./g.)
Potentiometric titration against $HClO_4$	—	0·995
Conductometric titration against $HClO_4$	0·94	1·02
Other methods	0·90,* 0·94†	‡

* Titration against HCl (Bowes & Kenten, 1948).
† Amino-acid analysis (Bowes & Kenten, 1948).
‡ Values for this sample of gelatin by other methods are not available, but results for other gelatins by titration against HCl are usually between 0·95 and 1·00, depending on the preparation.

SUMMARY

A method is described for estimating the total basic groups of collagen and gelatin by direct titration with perchloric acid, using anhydrous formic and acetic acids as solvents throughout. The end point can be satisfactorily determined by potentiometric or conductometric methods. An accuracy of ± 1 % can be obtained, and the results compare favourably with those obtained by other methods. The advantage of this method is that it can be carried out on 1 g. of protein and is rapid.

Our thanks are due to the Director and Council of the British Leather Manufacturers' Research Association for permission to publish this work.

REFERENCES

Bowes, J. H. & Kenten, R. H. (1948). *Biochem. J.* **43**, 358.
Bowes, J. H. & Moss, J. A. (1951). *Nature, Lond.*, **168**, 514.
Fraenkel-Conrat, H. L. & Cooper, M. (1944). *J. biol. Chem.* **154**, 238.
Hall, N. F. & Conant, J. B. (1927). *J. Amer. chem. Soc.* **49**, 3047.
Harris, L. J. (1935). *Biochem. J.* **29**, 2820.
Pankhurst, K. G. A. (1949). *Surface Chemistry*, p. 118. London: Butterworth.
Sanger, F. (1945). *Biochem. J.* **39**, 507.